

# **Recombinantly Engineered Proteins Containing Cholera Toxin B Subunit as a Functional and Structural Element**

**Yanshuang Zou**

**A dissertation submitted to  
Kochi University of Technology  
in partial fulfillment of the requirements  
for the degree of**

**Doctor of Philosophy**

**Graduate School of Engineering  
Kochi University of Technology  
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**September 2006**

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**Special Course for International Students  
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# Abstract

Cholera toxin consists of one A subunit (CTA) and five B subunits (CTB). CTA contains the toxic domain CTA<sub>1</sub> as well as a short sequence CTA<sub>2</sub>. Toxic CTA<sub>1</sub> is responsible for the symptoms of cholera toxin. CTA<sub>2</sub> serves to link A subunit noncovalently to B subunit by fitting part of itself within the central pore formed by CTB pentamer. CTB mediates the binding of the holotoxin to GM<sub>1</sub> ganglioside receptors on mucosal epithelia.

It was elucidated that B subunit was very useful as an adjuvant and carrier to increase the immunogenicity of vaccines and antigens coupled to it. In addition, a novel strategy to construct the holotoxin-like complex consisting of antigen peptide-CTA<sub>2</sub> and pentameric CTB, is also attractive. This strategy utilized CTB as a transporter for the effective delivery of vaccines and antigens with reduced toxicity and high efficacy.

All these confer CTB a bright prospect as a vehicle to facilitate the induction of mucosal immune responses for a broad range of vaccines and antigens for human purposes.

## Part I

### Utilization of CTB as an adjuvant and carrier:

#### Expression of recombinant antigens of Japanese cedar pollen allergen T cell epitopes fused with CTB in *Bacillus subtilis*, aiming at the treatment of Japanese cedar pollinosis

Peptides only containing T cell epitopes from allergens, which are not reactive to the allergen-specific IgE, are appropriate candidates as antigens for the prevention and treatment of allergy. As many T cell epitopes from allergens as possible should be included to achieve sufficient efficacy in a large population of allergic patients. Japanese cedar (*Cryptomeria japonica*) pollen is one of the most prevalent allergens in Japan. Two major allergenic proteins of the pollen, Cry j1 and Cry j2, have been isolated and characterized. In the study, up to five major T cell epitopes from Cry j1 and seven from Cry j2 were selected on the basis of references. To utilize the ability of CTB as the adjuvant and carrier, genes encoding epitopes from Cry j1 or Cry j2 were fused to CTB gene in tandem by PCR, to construct two fusion genes,



*ctb-cry j1 epi* and *ctb-cry j2 epi*. The fusion genes were expressed firstly in *E. coli*. The expressed CTB-Cry j1 epi and CTB-Cry j2 epi were purified to a high homogeneity with Ni-NTA resin, giving about 11 and 18 mg/L culture of purified proteins, respectively. Results of antigenic reactivity by western blotting showed that recombinant CTB-Cry j1 epi or CTB-Cry j2 epi was recognized specifically by anti-cholera toxin as well as anti-Cry j1 (or anti-Cry j2) antibodies. It was found that Cry j1 epi and Cry j2 epi could not be expressed without CTB fused to them, indicating that CTB also functioned to stabilize short epitopes expressed in bacteria-based systems.

A traditional Japanese food, natto, is an ideal food carrier for edible antigens and vaccines, especially for the treatment of Japanese cedar pollinosis, because it is made easily and full of nutrients, and most importantly, is the favorite food of Japanese people. Natto is made from soybeans fermented by a bacterium classified as *Bacillus subtilis* (*natto*). Analysis of genome showed the structure of *B. subtilis* (*natto*) strain was similar to that of *Bacillus subtilis*. It was demonstrated that repeated transformation of a *B. subtilis* strain with genomic DNA from *B. subtilis* (*natto*) conferred *B. subtilis* the ability to ferment natto. On the other hand, the non-cognate DNA fragment can be accommodated in the genome of *B. subtilis* through homologous recombination of pBR322-derived sequences artificially integrated in the *B. subtilis* genome. Therefore, natto fermented by the hybrid *B. subtilis* containing the antigen gene in its genome becomes a therapeutic agent of the allergy.

In the study, in order to try to express the antigen in *B. subtilis*, the gene of *ctb-cry j1 epi* was firstly inserted in the vector pHASH120 which contains *E. coli* plasmid pBR322-derived sequences. The constructed plasmid was named as pHASH120-*ctb-cry j1 epi*. The pBR322-derived sequences were also preinstalled in the genomic *leuB* locus of *B. subtilis* strain 168 *trpC2* to construct *B. subtilis* strain BEST2131. BEST2131 accepted the gene of *ctb-cry j1 epi* by homologous recombination after being transformed by the constructed plasmid. Although results revealed the successful insertion of *ctb-cry j1 epi* in the genome of BEST2131, the expression of CTB-Cry j1 epi from *ctb-cry j1 epi* gene in the genome of BEST2131 was not detected. To verify whether the antigen gene is functional or not, a multi-copy plasmid pGETS103 with the full length of pBR322 was utilized to recover *ctb-cry j1 epi* from the genome of BEST2131. The resultant pGETS103 containing *ctb-cry j1 epi* was employed to

transform *B. subtilis* 168 *trpC2*, which does not possess pBR322-derived gene, and thus no homologous recombination happens between the plasmid and the genome. The transformant was shown to produce much quantity of the antigen peptide. This indicated that the recovered antigen gene was functional in the plasmid and the antigen protein was successfully expressed in *B. subtilis*. It was assumed that the undetectable expression of the antigen peptide from the gene in the genome might be due to: (1) single copy number of the antigen gene in the genome; (2) some unknown factors in the genome which affected the normal expression of the inserted gene; (3) insufficient sensitivity and specificity of western blotting to detect antigen protein if there was expression.

Middle wall protein (MWP) promoters from *Bacillus brevis* 47 are stronger promoters for the transcription of mRNA, therefore the stronger expression of proteins. However, even if MWP promoters were used as the promoter of the antigen gene, also no detection of the antigen peptide in the genome was available. Complex factors in the genome for the expression of the foreign protein should be investigated.

## **Part II**

### **Utilization of CTB as a transporter:**

#### **Expression of pentameric CTB and an attempt of the assembly of holotoxin-like complex in a cell-free system**

A new strategy for the delivery of antigens is to co-express CTB and the interested peptide or protein linked to the nontoxic CTA<sub>2</sub> segment to create a holotoxin-like chimera. This generates CT-based mucosal vaccines or antigens with reduced toxicity and high efficiency. However, to our knowledge, the recombinant CTB expressed in *E. coli* was in an insoluble form, therefore impossible to assemble holotoxin-like complex. The only *in vitro* assembly method involved denaturation and renaturation of CTA<sub>2</sub>-fusion protein and CTB, which was troublesome and actually difficult for the practical application. The cell-free system can provide the platform to assemble the holotoxin-like complex by co-expressing CTB and the fusion protein of interested peptide or protein linked to CTA<sub>2</sub> simultaneously. However, before the assembly of the holotoxin-like complex is carried out, the ability of CTB to form the pentamer has to be

verified.

Results showed that pentameric CTB was little expressed in *E. coli*, but was expressed in the cell-free system easily and steadily. This indicated that the cell-free system was a valuable tool and platform for the expression of structurally complex oligomeric proteins.

An attempt to assemble cholera toxin-like complex by co-expressing CTB and the fusion protein of green fluorescence protein (GFP) linked to CTA<sub>2</sub> in the cell-free system was performed. Results revealed no formation of complex. It was presumed that the incomplete expression of GFP-CTA<sub>2</sub> or burying of hydrophobic CTA<sub>2</sub> inside the hydrophobic region of the cylindrical structure of GFP, might result in the failure of the interaction between CTA<sub>2</sub> and CTB, thus the formation of holotoxin-like complex. SDS-PAGE results proved the interaction between the segments of GFP and CTA<sub>2</sub> in GFP-CTA<sub>2</sub>, although the kind of interaction remained unknown. More stable and/or more hydrophilic proteins or a flexible linker between GFP and CTA<sub>2</sub> are expected to be workable. The functional chaperones may facilitate and/or stabilize the formation of complex.

# Chapter 1

## General introduction

Cholera toxin (CT) is an oligomeric protein with the molecular weight of 84,000 daltons and consists of a single A subunit (CTA) surrounded by five B subunits (CTB). CTA contains the toxic domain (CTA<sub>1</sub>) as well as a short sequence (CTA<sub>2</sub>). CTA<sub>2</sub> serves to link A subunit noncovalently to B subunit by fitting part of itself within the central pore formed by CTB pentamer [1]. The schematic structure of cholera toxin is shown in Figure 1.1.

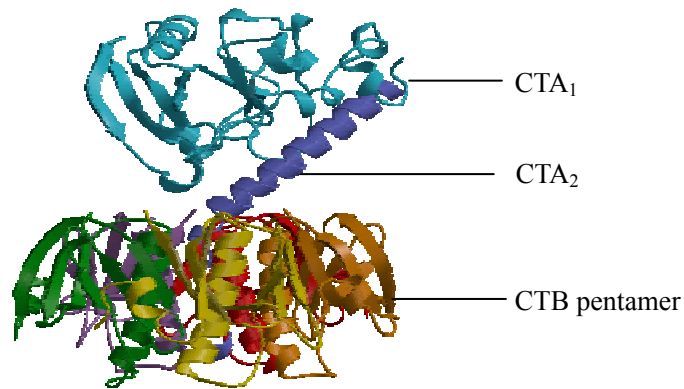


Figure 1.1 Schematic structure of cholera toxin

(<http://www.rcsb.org/pdb/smartSubquery.do?smartSearchSubtype=TreeQuery&t=11&n=56411>)

CT is a potent activator of adenylate cyclase and pathogenic agent responsible for the symptoms of cholera. The action mechanism of cholera toxin is shown in Figure 1.2. When cholera toxin is released from the bacteria in the infected intestine, it binds to the intestinal cells known as enterocytes through the interaction of the pentameric B subunit of the toxin with the GM<sub>1</sub> ganglioside receptor on the intestinal cell, triggering endocytosis of the toxin. Subsequently, the A/B cholera toxin must undergo cleavage of the A<sub>1</sub> domain from the A<sub>2</sub> domain in order for A<sub>1</sub> to become an active enzyme, i.e. ADP-ribosyltransferase. Once inside the enterocyte, the enzymatic A<sub>1</sub> fragment of the toxin A subunit enters the cytosol, where it activates the G protein G<sub>s</sub> through an ADP-ribosylation reaction that acts to lock the G protein

in its GTP-bound form, thereby continually stimulating adenylate cyclase to produce cAMP. The high cAMP levels activate the cystic fibrosis transmembrane conductance regulator (CFTR), causing a dramatic efflux of ions and water from infected enterocytes, leading to watery diarrhoea.

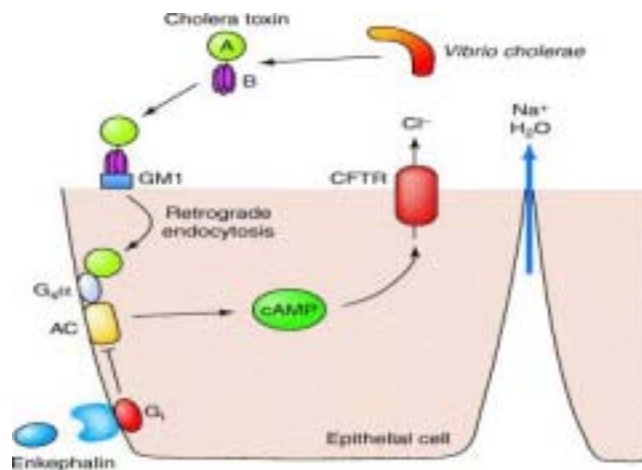


Figure 1.2 Action mechanism of cholera toxin

([http://www.ebi.ac.uk/interpro/potm/2005\\_9/Page2.htm](http://www.ebi.ac.uk/interpro/potm/2005_9/Page2.htm))

Cholera toxin has become a powerful research tool not only in microbiology, but in the fields of physiology, cell biology and biochemistry, as well. Cholera toxin has several immunomodulating effects which alone or in combination might explain its strong adjuvant properties in stimulating immune responses to admixed unrelated antigens and the subsequent appearance of corresponding mucosal IgA after oral immunization, although the mechanism underlying it remains unclear. CTB is an effective oral immunizing agent, which in a large field trial has been shown to afford protection against both cholera and enterotoxigenic *E. coli*-caused diarrhea. This has made CTB as such an important component, together with killed whole vibrios, of an oral cholera vaccine [2]. Moreover, CTB has attracted much interest recently as an adjuvant and immunogenic carrier for various other peptide antigens admixed or coupled to it, despite some researchers thought the coadministration of a little CTA was needed. Much progress has been made in preparing immunogenic hybrid antigens by coupling them chemically or genetically to CTB [3-6]. Indeed, in several systems, oral administration of such

hybrid antigens has been found to markedly potentiate both intestinal and extraintestinal IgA immune responses against the CTB-coupled antigens and also to elicit substantial circulating antibody responses. Therefore, CTB as an adjuvant and carrier is useful to improve the mucosal immunogenicity of antigens. It was also found that CTA<sub>2</sub> domain itself was necessary and sufficient to enable peptides linked to the N-terminus of CTA<sub>2</sub> to form stable holotoxin-like chimeras [7]. Thus, the construction of holotoxin-like complexes, utilizing CTB as a transporter for the effective delivery of antigens, is also desirable. This strategy provides the bright prospect for generating CT-based mucosal vaccines and antigens with reduced toxicity and high efficacy.

In summary, all these give promise for the use of CTB as vehicles to facilitate the induction of mucosal immune responses to a broad range of antigens for human vaccination purposes.

## Chapter 2

### **Utilization of CTB as an adjuvant and carrier for oral delivery of antigen peptides:**

### **Expression of recombinant antigens of Japanese cedar pollen allergen T cell epitopes fused with CTB in *Bacillus subtilis*, aiming at the treatment of Japanese cedar pollinosis**

#### **2.1 Introduction**

One of promising approaches to the prevention and treatment of allergy is desensitization by vaccination with peptides which are derived from allergens. To avoid allergic reactions due to the presence of allergen-specific IgE binding sites in the whole antigen, the novel antigen lacking the epitopes reactive to IgE should be utilized. Peptides only containing T cell epitopes from allergens are appropriate candidates as antigens. Whereas, a major difficulty with this approach is the diversity of MHC class II molecules in individuals, resulting in patients with different MHC class II molecules responding to different allergen-derived peptides [8]. Therefore, as many T cell epitopes of allergens as possible should be included to achieve sufficient efficacy in a large population of allergic patients [9]. Japanese cedar pollen, produced largely in spring, is one of the most prevalent allergens in Japan. Japanese cedar pollinosis has become a national disease in Japan, with more than 10% of the population suffering from it [10]. Two major allergenic proteins of the pollen, Cry j1 and Cry j2, have been isolated and characterized [11-13]. Recombinant peptides, in which multiple T-cell epitopes from Japanese cedar allergenic proteins were linked, have been developed, and basic immunologic studies have exhibited their potency as immunotherapeutic agents [9, 14, 15]. However, not many T cell epitopes of Cry j1 and Cry j2 were included in the developing and developed immunotherapeutic peptides. In the study, much more epitopes, i.e. five major T-cell epitopes from Cry j1 and seven from Cry j2 were selected on the basis of references [9, 14].

Much progress has been made in the utilization of non-toxic cholera toxin B subunit (CTB) as an adjuvant and antigen-carrier system to enhance the mucosal immune response to various foreign antigens chemically or genetically coupled to it. In the study, genes encoding epitopes from Cry j1 or Cry j2 were fused to CTB gene in tandem by PCR, to construct two fusion genes, *ctb-cry j1 epi* and *ctb-cry j2 epi*.

Edible vaccines and antigens do not accompany the torment by repeated injections and the refrigeration is not needed for storage. Particularly, the availability of vaccines and antigens at a reasonable cost by the mass production is valuable for developing countries. Thus, the development of edible vaccines and antigens is very attractive. The expression of vaccines and antigens in plants (such as potato, rice and etc), algae and edible bacteria (such as *Bacillus subtilis*) is practical in obtaining the mass production of edible vaccines and antigens. Recently, plantation of transgenic rice preventing Japanese cedar pollinosis is being under plan in Japan. However, the possibility of gene diffusion to surrounding plants by pollination limits the application of vaccine or antigen-producing transgenic plants. Edible bacteria and algae are outstanding with advantages such as easy cultivation and short harvest period. A traditional Japanese food, natto, was fermented from soybeans by the bacterium named as *B. subtilis* (*natto*). Natto is an ideal food carrier for edible antigens and vaccines, especially for the treatment of Japanese cedar pollinosis, because it is easily made and full of nutrients and can be stored with the fungicidal antibiotics produced by the natto-producing bacterium, and most importantly, is the favorite food of Japanese people.

*B. subtilis* (*natto*) is classified as closely related strain to *B. subtilis* strain 168 *trpC2*, which is the best-characterized gram-positive bacterium [16]. Analysis of genomic structure showed the structure of *B. subtilis* (*natto*) was roughly 98% similar to that of *B. subtilis* 168 *trpC2* and the only 2% difference might account for their biochemical divergence [17, 18]. Extensive biochemical and genetic studies at a molecular level have not been conducted on the genes and enzymes involved in the fermentation of natto [19-23], although the genes required for a natto production bioprocess have been yet to be totally clarified. A limited number of genetically and biochemically characterized gene homologues, including *psgABCD* involved in the synthesis of  $\gamma$ -PGA, *iep* involved in the regulation of protease secretion, and *glr* and *yrcP* involved in the racemization of glutamate, are also present in the genome of *B. subtilis* 168 *trpC2*. However, *B.*



*subtilis* 168 *trpC2* does not produce capsular PGA, suggesting that highly coordinated regulation of gene expression, as well as physiological conditions during growth on the soybean surface, are required for good natto producers. The gene regulation of natto fermentation can function in *B. subtilis* 168 *trpC2* if the relevant genes are appropriately transferred to corresponding loci of the genome via homologous recombination by repeated transformation of *B. subtilis* 168 *trpC2* with the genomic DNA from *B. subtilis* (*natto*) [17]. As more DNA sequence from the natto strain was transferred to the recipient *B. subtilis* 168 *trpC2* strain, the natto fermentation ability was gradually enhanced in proportion to the amount of the natto strain-derived DNA. It was found that the assembly of >300kb segments from *B. subtilis* (*natto*) in the genome of *B. subtilis* 168 *trpC2* converted the strain 168 *trpC2* to a *natto* producer [24, 25].

*B. subtilis* 168 *trpC2*, has several genetic features that distinguish it from the well-known gram-negative *E. coli*, and has historically been used as a recipient for horizontal transfer of genes due to its ability to develop competency [26]. To circumvent the potential obstacles accompanying the use of plasmid vectors, i.e. variable copy number per cell and genetic instability, the creation of strains carrying interested gene sequence stably maintained in the *B. subtilis* genome is an alternative strategy. The non-cognate DNA can be inserted in the genome of *B. subtilis* by homologous recombination, which happens between two homologous DNA segments, one in the chromosome and the other in the plasmid containing the interested gene. pBR322 has many advantages as an artificially integrated sequence in the *B. subtilis* genome for the homologous recombination [26]: (1) its entire nucleotide sequence has been determined; (2) its replication profile in *E. coli* has been well established; (3) it does not replicate autonomously in *B. subtilis*; (4) two marker genes for *E. coli*, the  $\beta$ -lactamase gene (ampicillin resistance) and the tetracycline resistance determinant gene (tetracycline resistance) are inert in *B. subtilis*; (5) several useful restriction enzyme sites are available; (6) it has a low copy number in *E. coli* (15-20 copies per cell); and (7) a cosmid vector pHC79, frequently used to clone large DNA segments, has a structure identical to that of pBR322 except for the *cos* sequence at the *PvuII* site. The integrated pBR322 sequence in the genome of *B. subtilis* can replicate as part of the chromosome and is used to accommodate the non-cognate DNA fragment.

A novel vector, named as pHASH120, was constructed by Ohashi *et al* [27]. Features of the

vector can be summarized as follows: (1) a promoter amplified by PCR can be introduced to the vector directly with the TA-cloning method; (b) an open reading frame (ORF) amplified by PCR can be oriented downstream of the promoter and RBS at an optimized distance; and (3) the newly constructed gene in *E. coli* is directly cloned into the *B. subtilis* genome as a single copy. The interested gene together with the promoter and RBS between the pBR322-derived sequences in the recombinant pHASH120 can be integrated into the genome of *B. subtilis* via pBR322 sequences carried by *B. subtilis* by homologous recombination. The vector uses the promoter and RBS optimal for *B. subtilis*, so optimal expression of inserted non-cognate DNA in the genome of *B. subtilis* is expected. Moreover, because the integrated DNA at the genomic pBR locus can be transferred to a multi-copy plasmid pGETS103 by a recombinational transformation procedure, the copy number of the cloned genes can be increased immediately.

In summary, the antigen gene inserted in pHASH120 can be inserted in the genome of *B. subtilis* by homologous recombination. The resultant *B. subtilis* with the antigen gene in the genome can obtain the ability in the fermentation of natto by repeated transformations with the genomic DNA of *B. subtilis* (*natto*), thus available for the preparation of edible antigen. The scheme about the preparation of edible antigen using natto as the food carrier is shown in Figure 2.1.

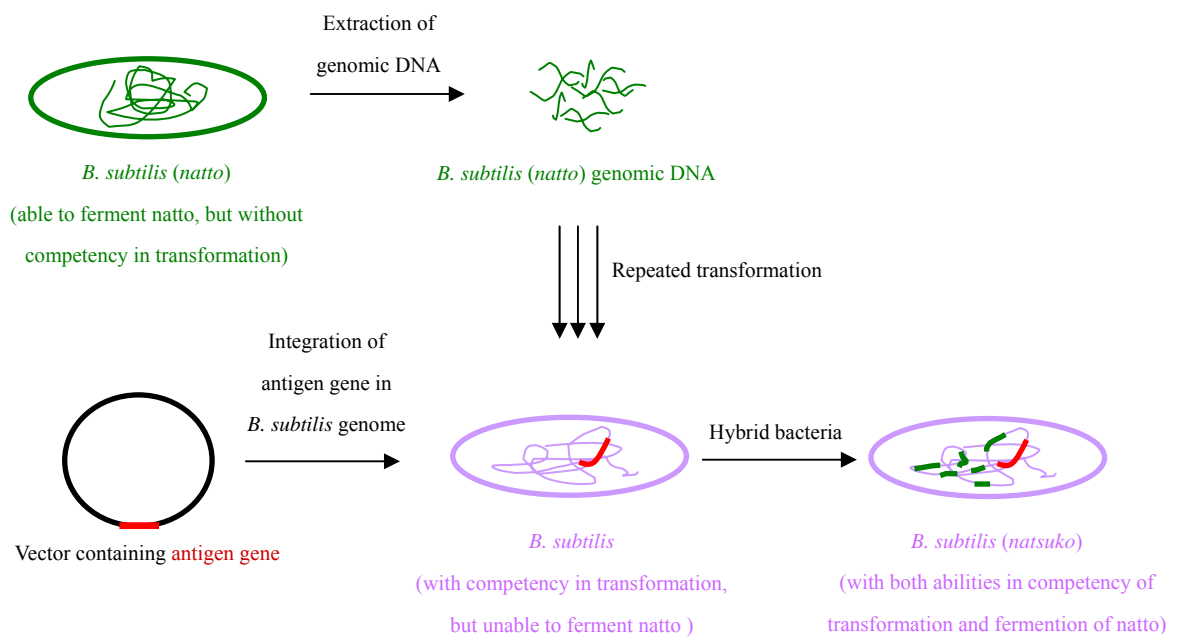


Figure 2.1 Scheme about the preparation of edible antigen using natto as the food carrier

## 2.2 Materials and methods

### 2.2.1 Bacterial strains, plasmids and media

Plasmids and bacterial strains used in the study are listed in Table 2.1, respectively.

Recombinant plasmids of pUC18 and pET28a (Novagen) were amplified using *E. coli* strain JM109 (Takara) as the host, and ampicillin (100µg/ml) and kanamycin (25µg/ml) were used as the selection antibiotics respectively. *E. coli* strain BL21 (DE3) (Novagen) was used for the expression of antigen peptides.

*B. subtilis* strains, including *B. subtilis* 168 *trpC2* and *B. subtilis* BEST2131, and plasmids, including pHASH120 and pGETS103 which are related to amplification and expression of the antigen gene in *B. subtilis*, were kindly gifted by Dr. Mitsuhiro Itaya of Mitsubishi Kagaku Institute of Life Sciences, Tokyo. BEST2131 was constructed from 168 *trpC2* (original strain), with the insertion of pBR322 sequences in the *leuB* gene (*leuB*::pBRTc) of 168 *trpC2* [26] and becomes resistant to tetracycline. *E. coli* strain DH5α (Takara) was used for amplification of recombinant plasmids of pHASH120 and pGETS103. To select positive transformants, 50µg/ml of ampicillin, 5µg/ml of chloramphenicol, 10µg/ml of tetracycline, and 250µg/ml of blasticidin S were used.

All the antibiotics were purchased from Wako, except ampicillin which was purchased from Sigma. *E. coli* and *B. subtilis* were grown at 37 °C in LB medium.

Table 2.1 Plasmids and bacterial strains used in the study

Plasmid (kb)	Description	Bacterial strain	Antibiotic resistance*	Purpose
pUC18 (2.7)	-	JM109	Amp <sup>R</sup>	Cloning
pUC18- ctb-cry j1 epi (3.2)	Containing <i>ctb-cry j1 epi</i>	JM109	Amp <sup>R</sup>	Cloning
pUC18- ctb-cry j2 epi (3.3)	Containing <i>ctb-cry j2 epi</i>	JM109	Amp <sup>R</sup>	Cloning
pET28a (5.4)	-	JM109	Kana <sup>R</sup>	Cloning
pET28a- ctb-cry j1 epi (5.9)	Containing <i>ctb-cry j1 epi</i>	JM109	Kana <sup>R</sup>	Cloning
		BL21 (DE3)	Kana <sup>R</sup>	Expression of antigen
pET28a- ctb-cry j2 epi (5.9)	Containing <i>ctb-cry j2 epi</i>	JM109	Kana <sup>R</sup>	Cloning
		BL21 (DE3)	Kana <sup>R</sup>	Expression of antigen
pHASH120 (5.8)	Containing PS10-RBS	DH5α	Bs <sup>R</sup> , Amp <sup>R</sup> and Cm <sup>R</sup>	Cloning
		<i>B. subtilis</i> BEST20038	Tc <sup>S</sup> , Bs <sup>S</sup> and Cm <sup>R</sup>	Negative control for antigen expression
pHASH120- ctb-cry j1 epi (6.3)	Containing PS10-RBS- <i>ctb-cry j1 epi</i>	DH5α	Bs <sup>R</sup> , Amp <sup>R</sup> and Cm <sup>R</sup>	Cloning
		<i>B. subtilis</i> BEST2131	Tc <sup>S</sup> , Bs <sup>S</sup> and Cm <sup>R</sup>	Integration of PS10-RBS- <i>ctb-cry j1 epi</i> in <i>leuB</i> loci of genome
pGETS103 (14.5)	Shuttle vector of <i>E. coli</i> and <i>B. subtilis</i>	DH5α	Amp <sup>R</sup> and Tc <sup>R</sup>	Cloning
<i>Hind</i> III digested pGETS103 (14.5)	Unable to self-replicate in <i>B. subtilis</i>	<i>B. subtilis</i> BEST2131- <i>ctb-cry j1 epi</i>	Tc <sup>R</sup> and Cm <sup>R</sup>	Recovery of antigen gene from genome to multi-copy plasmid
pGETS103- ctb-cry j1 epi (15.0)	Containing PS10-RBS- <i>ctb-cry j1 epi</i>	DH5α	Cm <sup>R</sup>	Cloning
		<i>B. subtilis</i> 168 <i>trpC2</i>	Tc <sup>R</sup> and Cm <sup>R</sup>	Investigation of antigen expression in multi-copy plasmid

\* Antibiotics indicated are: Amp, ampicillin; Kana, kanamycin; Cm, chloramphenicol; Tc, tetracycline; Bs, blasticidin. S<sup>R</sup> and S<sup>S</sup> refer to “resistant” and “sensitive”, respectively.

### 2.2.2 Selection of major T cell epitopes from Cry j1 and Cry j2

On the basis of references, major T cell epitopes from Cry j1 and Cry j2 were selected. Selection based on considerations that epitopes should be able to stimulate strong proliferative response of T cell line which is Cry j1 or Cry j2-specific, and various MHC class II types, the molecules of which are capable of presenting T cell epitopes, should be included to obtain reliable applicability in a large population of patients.

### 2.2.3 Construction of fusion genes

Fusion genes of *ctb-cry j1 epi* and *ctb-cry j2 epi* were acquired by stepwise PCR: *ctb* was used as the template of the first PCR and the resultant PCR product was used as the template of the next reaction, and other reactions were performed in the same way. The forward primer (5'actgc**ggatccacacctcaaatattactg**3', the sequence indicated in bold is the *Bam*HI site, and the sequence written in red characters is complementary to the beginning coding sequence of *ctb*) was designed to give rise to the *Bam*HI site at the 5' end. The reverse primer from P1-P5 or P'1-P'7 possesses an overlapping sequence homologous to the template and an overhang of the epitope gene which was devised based on the codon usage in *E. coli*.. Sequences and the reaction order of reverse primers are listed in Table 2.2 and 2.3. Since it is difficult and costly to synthesize the primer having long gene sequence of peptide such as Cry j1-106-125, Cry j2-191-209 or Cry j2-345-374, these genes were divided into two separate primers. Finally, the reverse primer of Cry j1-212-224-*Hind* III (5'gcgt**caagcttcgggccgaactgggtgaac**3', the sequence indicated in bold is the *Hind* III site, and the sequence written in red characters is complementary to the template) or Cry j2-345-374-2-*Hind* III (5'gctgaa**agcttgaaatagccgttcgcgtta**3', the sequence indicated in bold is the *Hind* III site, and the sequence written in red characters is complementary to the template) was used to produce the *Hind* III site at the 3' end of the fusion gene of *ctb-cry j1 epi* or *ctb-cry j2 epi*.

Table 2.2 Reverse primers used to construct the fusion gene of *ctb-cry j1 epi*

Name	Position in Cry j1 antigen	Sequence* (5'-3')
P1	Cry j1-16-30	gctgccgaagcccaccgcgcaatccgccagtttcacaggttctgattgccataactaattgcgg
P2	Cry j1-81-95	acggccatcgaacgtttatagcccgaatatacatcgccatttctgtgccgaagcccac
P3	Cry j1-106-125-1	gttgctcacacgtttaatgaacacgcacggacggccatcgaacgtttat
P4	Cry j1-106-125-2	gccatacagatgcaggccatgaataatcacgttgctcacacgtttaatga
P5	Cry j1-212-224	cgggccgaactggtgaacgccaccgtcactttcatgctgcatacagatgcaggccat

\* The sequence written in red characters is complementary to the template.

Table 2.3 Reverse primers used to construct the fusion gene of *ctb-cry j2 epi*

Name	Position in Cry j2 antigen	Sequence* (5'-3')
P'1	Cry j2-77-89	ttccagctcgccgggttctgatacgcgcgaataatgccattgccataactaattgcgg
P'2	Cry j2-96-107	gcccatcagcgtgaagcccgtcagtttcgcgaactgttccagctcgccgggttct
P'3	Cry j2-191-209-1	atggaagttttgctcgcgaaaataatcaatgcccatcagcgtgaagccc
P'4	Cry j2-191-209-2	gcccggtccaatcgtgttttctgcagatggaagttttgctcgcga
P'5	Cry j2-245-259	gaatttcgcgcgttcacatgcacatagctcacttcgcacggctgccgtgccaatcgt
P'6	Cry j2-345-374-1	gctcgtcagtttcaggctaataatcgtcagtttaataatctttgcaaatcgcgcgtt
P'7	Cry j2-345-374-2	gaaatagccgttcggttatcgttcaggcagctcgcaatttggcgtcgtcagtttcag

\* The sequence written in red characters is complementary to the template.

All primers were purchased from Proligo. PCR was accomplished with high fidelity KOD DNA polymerase (Toyobo). PCR reaction conditions were as follows: 98 °C, 5min preheating, followed by 25 cycles of denaturing (98 °C, 15sec), annealing (65 °C, 2sec) and elongation (74 °C, 30sec). After each step of PCR, the product was purified using PCR Purification Kit (Qiagen) or purified from the agarose (Takara) gel using Gel Extraction Kit (Qiagen) according to manufacturers' instructions. PCR products were analyzed on the 2% agarose gel stained with ethidium bromide by electrophoresis.

## 2.2.4 Construction of recombinant plasmids for *E. coli*

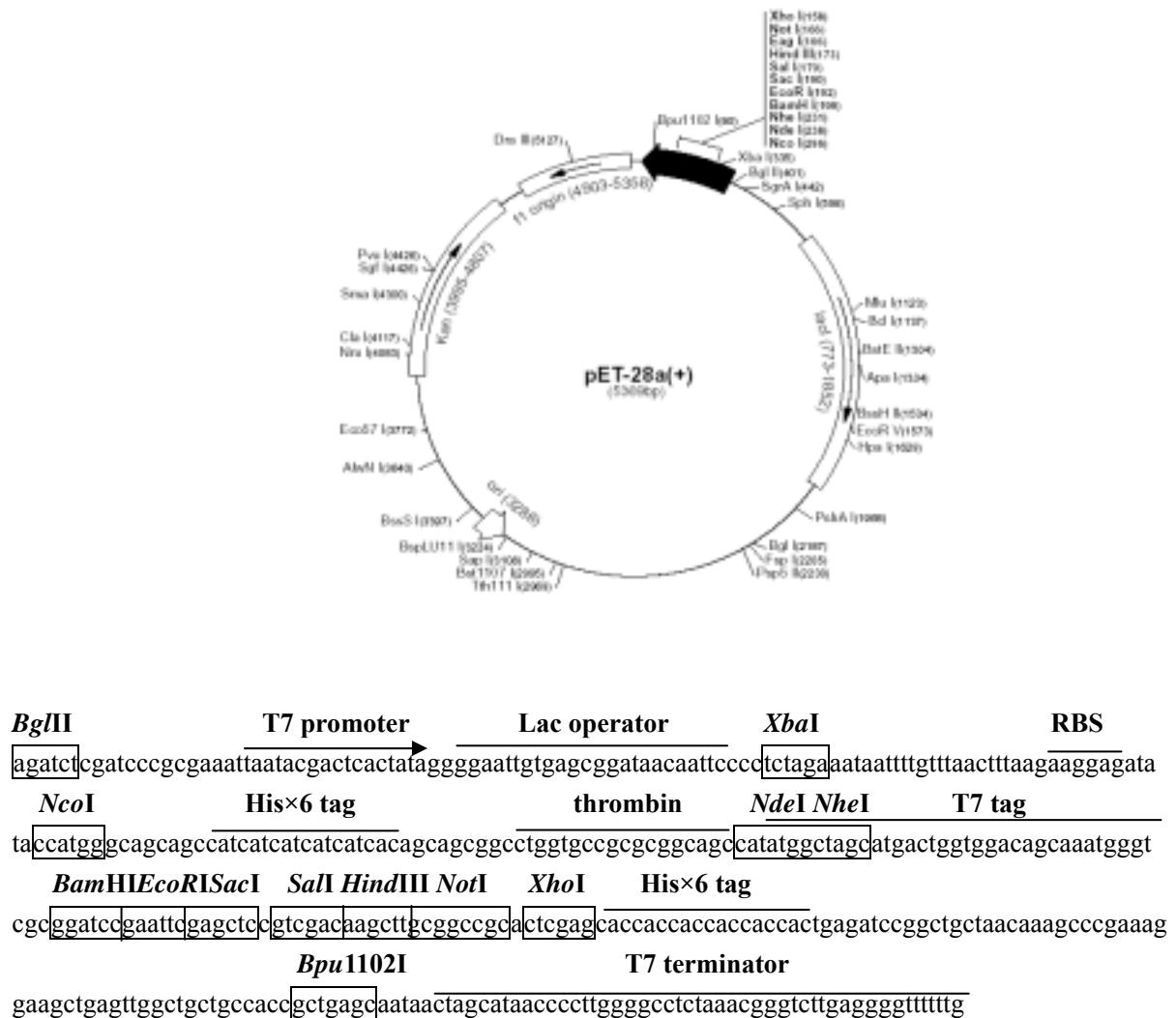


Figure 2.2 Structure and cloning/expression region of *E. coli* plasmid pET28a

([http://www.genomex.com/vector\\_maps/pET28\\_map.pdf](http://www.genomex.com/vector_maps/pET28_map.pdf))

The resultant DNA fragments of *ctb-cry j1 epi* and *ctb-cry j2 epi* from PCR were digested with *Bam*HI and *Hind* III restriction enzymes (Takara) and cloned into corresponding sites of pUC18 plasmid to produce recombinant cloning plasmids of pUC18-*ctb-cry j1 epi* and pUC18-*ctb-cry j2 epi*. *E. coli* JM109 competent cells were transformed with the recombinant cloning plasmids, and positive colonies were selected from LB/ampicillin plates. Inserts were digested out of extracted plasmids with *Bam*HI and *Hind* III restriction enzymes, and then

cloned into pET28a plasmid, which provides two 6×his-tag coding sequences at both termini for one-step purification of expressed proteins, to construct recombinant expression plasmids of pET28a-ctb-cry j1 epi and pET28a-ctb-cry j2 epi. The structure and the cloning/expression region of pET28a are shown in Figure 2.2. Then, *E. coli* BL21 (DE3) competent cells were transformed with the expression plasmids, and positive colonies selected from LB/kanamycin plates were used in the expression and purification procedure. All the enzymatic digestion products were purified from the agarose gel using Gel Extraction Kit (Qiagen).

DNA sequences of *ctb-cry j1 epi* and *ctb-cry j2 epi* in recombinant cloning plasmids and the orientations of the inserts in recombinant expression plasmids were analyzed using Thermo Sequenase Cy5.5 Dye Terminator Cycle Sequencing Kit (Amersham pharmacia).

#### 2.2.5 Construction of recombinant plasmids for *B. subtilis*

##### 2.2.5.1 Recombinant plasmid used for the insertion of the antigen gene in the genome of *B. subtilis*

The structure of pHASH120 is shown in Figure 2.3. Upstream of RBS, *Sma*I is positioned for the cloning of optional promoters using the T-extension cloning method, and PS10 was used as the promoter in the study because it was proved one of the strongest promoters in *B. subtilis*. An interested gene amplified by PCR can be cloned in the T-extended *Eco*RV ends. The ampicillin-resistant and the part of tetracycline-resistant ( $\Delta$ tet) genes are derived from pBR322 vector, the sequence between them can be integrated in the genome of *B. subtilis* BEST2131 by homologous recombination.

T vector was prepared according to the method described by Ohashi *et al* [27]. Two  $\mu$ g pHASH120 was digested completely with 30U of *Eco*RV (Takara) at 37 °C for 6h and treated with phenol/chloroform extraction. After precipitation with ethanol, DNA was dissolved in 10 $\mu$ l of TE buffer. An aliquot of 5 $\mu$ l of the digested plasmid solution was treated with *Ex Taq* DNA polymerase (Takara) at a ratio of 2.5U/ $\mu$ g plasmid in 50 $\mu$ l buffer solution (1  $\times$  *Ex Taq* buffer supplemented with 2mM dTTP) at 72 °C for 2h. After phenol/chloroform extraction twice and isopropanol precipitation, the T-vector was prepared by dissolving in 10 $\mu$ l of TE buffer and



The diagram illustrates the pHASH120 vector, a 5386bp circular plasmid. Key features include:

- Promoter (PS10):** Located at the top left, with sequences CCCT AGGG and GGGA TCCC.
- ORF (ctb-cryj1 epi):** Located at the top right, with sequences GATT AATC and CTA A TTAG.
- Insertion Site:** A box labeled 'RBS' (Ribosome Binding Site) and 'aaattGATATC' is shown, flanked by 'SmaI' and 'EcoRV' sites.
- Antibiotic Resistance Genes:** Amp (ampicillin), Cm (chloramphenicol), ΔTc (part of tetracycline-resistant gene), and Bs (blasticidin S).
- Restriction Enzyme Sites:** SmaI, EcoRI, XbaI, BglII, PstI, and SalI are indicated on the plasmid map.

Figure 2.3 Schematic structure of pHASH120 vector and the insertion site of the antigen gene

Antibiotic-resistance genes indicated are: Amp, ampicillin; Cm, chloramphenicol; ΔTc, part of tetracycline-resistant gene; Bs, blasticidin S

(Ohashi Y, et al [27]. Far different levels of gene expression provided by an oriented cloning system in *Bacillus subtilis* and *Escherichia coli*. FEMS Micro Lett. 2003;221:125-130.)

The gene of *ctb-cry j1 epi* was amplified by primers of CTB-FW (5' **atg**acacctcaaaatattac3', the bolded sequence is the start codon) and Cry j1-RV (5' **ttac**gggccgaactggttga3', the bolded sequence is the stop codon) from plasmid pUC18-ctb-cry j1 epi using *Ex Taq* DNA polymerase. PCR reaction conditions were as follows: 94 °C, 5min preheating, followed by 35 cycles of denaturing (94 °C, 15sec), annealing (55 °C, 15sec) and elongation (72 °C, 30sec), and an additional 7min reaction at 72 °C to add a dA nucleotide on both ends of the PCR product for the ligation with the T vector. The reaction of 100ng of the T-vector with 10-fold molar excess of purified PCR product was carried out at 16 °C for at least 6h using DNA Ligation Kit Ver.1 (Takara) according to the method described in the manual.

The prepared recombinant plasmid named pHASH120-ctb-cry j1 epi was amplified in *E. coli* DH5 $\alpha$ . Because of the possibility of two different orientations of *ctb-cry j1 epi* in the T-extended *EcoRV* ends of pHASH120, primers of *EcoRI-SmaI*-RBS-FW (5'ccggaattcccccgggaaaggaggaaattga3') and Cry j1-RV were used to search for the recombinant plasmid with the desired orientation of the antigen gene. Because *EcoRI-SmaI*-RBS-FW primer has corresponding sequence of RBS, but not that of *ctb*, the insert with unwanted orientation can not be amplified.

The obtained recombinant plasmid with the desired orientation of the antigen gene was used for the transformation of *B. subtilis* BEST2131 to insert the antigen gene in the genome of the bacterium. The obtained positive strain was named as BEST2131-ctb-cry j1 epi. The genomes were extracted using QIAamp DNA mini Kit (Qiagen). Primers of CTB-FW and Cry j1-RV were used to confirm the insertion of *ctb-cry j1 epi* in the genome.

#### 2.2.5.2 Multi-copy plasmid used for the recovery of the antigen gene from the genome of *B. subtilis*

The structure of pGETS103 is shown in Figure 2.4. The vector of pGETS103 consists of pTB522, a  $\theta$ -type replicating plasmid in *B. subtilis* and the full length of *E. coli* plasmid pBR322, and can shuttle between *E. coli* and *B. subtilis*. It has two antibiotic resistance genes from pBR322 (ampicillin resistance and tetracycline resistance, which are only functional in *E. coli*), and one from pTB522 (tetracycline resistance, which is only functional in *B. subtilis*). Between pBR322-derived antibiotic resistance genes, there is a *Hind* III site, which was used for the linearization of the vector.

The vector of pGETS103 was linearized prior to transformation. The linearized pGETS103 is unable to self-circularize, and circular pGETS103 can be established only after recovering the antigen gene from the genome by the homologous recombination mechanism. Linearized pGETS103 was prepared by digesting 1 $\mu$ g of pGETS103 with 10U of *Hind* III at 37 °C for 2h, followed by phenol/chloroform extraction and ethanol precipitation. The linearized pGETS103 was used to transform *B. subtilis* BEST2131-ctb-cry j1 epi to recover the antigen gene from the genome by homologous recombination.

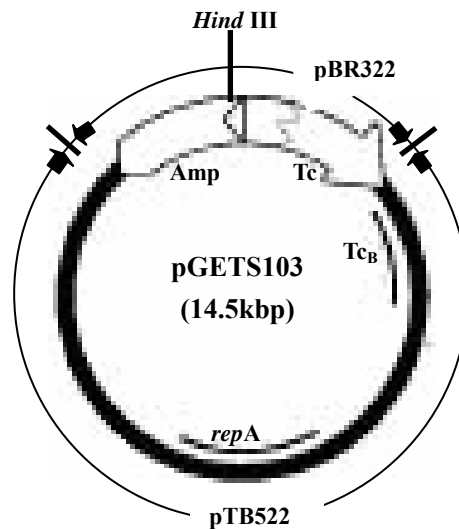


Figure 2.4 Schematic structure of pGETS103 vector

Antibiotic-resistance genes indicated are: Amp, ampicillin; Tc, tetracycline-resistant gene, only functional in *E. coli*; Tc<sub>B</sub>, tetracycline-resistant gene, only functional in *B. subtilis*.

(Tsuge K and Itaya M [28]. Recombinational transfer of 100-kilobase genomic DNA to plasmid in *Bacillus subtilis* 168. J Bacteriol. 2001;183:5453-5458.)

The vector of pGETS103 containing the antigen gene was recovered from the positive transformant. Primers of CTB-FW and Cry j1-RV were used to confirm the recovery of *ctb-cry j1 epi* in the plasmid. The recovered recombinant plasmid of pGETS103-ctb-cry j1 epi was amplified in *E. coli* DH5α (20 copy number in *E. coli* compared with 9 in *B. subtilis*), and used for the transformation of *B. subtilis* 168 *trpC2*, which has no pBR322 sequences in the genome. The expression of CTB-Cry j1 epi was performed to test whether the expression of antigen gene was functional or not.

## 2.2.6 Transformation method

### 2.2.6.1 Transformation of *E. coli*

Solution after plasmid ligation (2μl) or plasmid (10ng) was incubated with competent *E. coli* cells (20μl) for 5min on ice, followed by heat shock with 42 °C for 30sec. After 2min on ice,

90µl SOC was added. Competent cells were grown at 37 °C under shaking for 1h and then applied on LB plate supplemented with an appropriate antibiotic, followed by incubation at 37 °C overnight.

Positive colonies transformed by recombinant plasmids of pUC18 or pET28a were selected on LB plates supplemented with ampicillin or kanamycin, respectively. Positive transformants transformed by pHASH120-ctb-cry j1 epi or pGETS103-ctb-cry j1 epi were selected on LB plate containing chloramphenicol or ampicillin.

#### 2.2.6.2 Transformation of *B. subtilis*

##### 2.2.6.2.1 Transformation of *B. subtilis* BEST2131 with the recombinant vector of pHASH120-ctb-cry j1 epi or linearized pGETS103

Solutions used for the transformation are listed in Table 2.4 (I) and (II).

A single colony of *B. subtilis* strain (BEST2131 or BEST2131-ctb-cry j1 epi) was inoculated in 1ml LB medium and grown under shaking at 37 °C for about 24h. The 24h-culture (50µl) and casamino acid (2%, 50µl) were added in TF-I (1ml), and the TF-I culture was continued to grow for another 4h under shaking at 37 °C. Then, 0.4ml of the TF-I culture was added in 3.6ml of TF-II and grown for 1h at the same condition. After the centrifugation at 4000rpm for 15min (4 °C), 175µl of TF-II and 75µl of glycerol (50%) were added to suspend the cell pellet to prepare competent cells. Competent cells were stored at -80 °C until use.

Competent cells (6.25µl), MgSO<sub>4</sub>· 7H<sub>2</sub>O (2%, 1.25µl) and MgCl<sub>2</sub> (1M, 1.25µl) were added in TF-D (50µl). Five µl of vector (pHASH120-ctb-cry j1 epi vector was used to transform *B. subtilis* BEST2131; and the linearized pGETS103 vector was used to transform *B. subtilis* BEST2131-ctb-cry j1 epi) was added and mixed well. The incubation was performed at 37 °C for 1h without shaking, followed by the addition of 200µl of LB medium and another 1h incubation at 37 °C under shaking.

Transformants (BEST2131 transformed by pHASH120-ctb-cry j1 epi vector) were selected on LB plate supplemented with tetracycline, blasticidin S or chloramphenicol by incubation at 37 °C overnight. Colonies which were only alive on LB plate supplemented with

chloramphenicol, were chosen as positive transformants. Transformants (BEST2131-ctb-cry j1 epi transformed by the linearized pGETS103 vector) were selected on LB/plate supplemented with tetracycline or chloramphenicol. Colonies grown on both plates were chosen as positive transformants.

Table 2.4 (I) Solutions used for the transformation of *B. subtilis*

Component	10×Spizizen	Medium A	Medium B
K <sub>2</sub> HPO <sub>4</sub>	140g	14g	
KH <sub>2</sub> PO <sub>4</sub>	60g	6g	
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	20g		2g
Tri-sodium citrate dihydrate (C <sub>6</sub> H <sub>5</sub> O <sub>7</sub> Na <sub>3</sub> • 2H <sub>2</sub> O)	10g		1g
H <sub>2</sub> O	to 1000ml	to 50ml	to 50ml

Table 2.4 (II) Solutions used for the transformation of *B. subtilis*

Component	TF-I (ml)	TF-II (ml)	TF-D (ml)
10×Spizizen solution	100	100	
Medium A			0.625
Medium B			6.25
50% glucose	10	10	1.25
2% MgSO <sub>4</sub> • 7H <sub>2</sub> O	10	10	
2% casamino acid	10	5	
5 mg/ml tryptophan	10	1	1.25
5 mg/ml arginine	10	1	1.25
5 mg/ml leucine	10	1	
5 mg/ml histidine			1.25
5 mg/ml <u>threonine</u>	10	1	
H <sub>2</sub> O	to 1000	to 1000	to 100

#### 2.2.6.2.2 Transformation of *B. subtilis* 168 *trpC2* with the recombinant vector of pGETS103-ctb-cry j1 epi

We found that the transformation method described above was not workable for the transformation of *B. subtilis* 168 *trpC2* with the recombinant vector of pGETS103-ctb-cry j1 epi. This was probably due to the low concentration of vector resulting from its low copy number. We adopted the electroporation method [29] with some modifications and obtained positive transformants.

*B. subtilis* 168 *trpC2* were firstly grown in 2ml of LB medium with vigorous shaking at 37 °C overnight. A 200ml LB medium was inoculated with the overnight culture and incubated at 37 °C under shaking until OD<sub>600</sub> reached 1.5-2.0. Cells were harvested by centrifugation at 3,000×g for 10min and washed three times (200ml/each time) in sterile cold ultrapure water and finally resuspended in 2ml of pre-chilled 30% polyethyleneglycol (PEG) 6000. The cells were then dispensed into 100µl aliquots, rapidly frozen in dry ice/ethanol and stored at -80 °C.

Electroporation was performed using the BTX electroporation system (Genetronisc). Cells (100µl) to be transformed were first thawed on ice, and 10µl of vector that had been dialyzed against deionized water was added, gently mixed and transferred to a pre-chilled 2mm gap electroporation cuvette. The cells were then immediately pulsed (2.5V, about 8msec of time constant at the resistance of 186Ω), diluted in 2ml of SOC medium and incubated at 37 °C with gently shaking (about 160rpm) for 1.5h. Chloramphenicol (0.2µg/ml) was added to the transformed cells in SOC after 1h incubation to induce resistance. Then 1ml of culture were centrifuged, and concentrated to 0.1ml, followed by being plated on LB plate.

Transformants were selected on LB plate supplemented with tetracycline or chloramphenicol by incubation at 37 °C overnight. Colonies growing on both plates were selected as positive transformants.

#### 2.2.7 Extraction of plasmids

Recombinant plasmids of pUC18 and pET28a from *E. coli* JM109 were extracted using Spin Miniprep Kit (Qiagen).

Recombinant plasmids of pHASH120-ctb-cry j1 epi and pGETS103-ctb-cry j1 epi have very low copy number due to the pBR322-derived origin and/or the large size. In order to obtain satisfactory concentrations, 50-400ml of LB culture was used for the growth of bacteria and the volume of the extracted plasmid in the elution buffer (EB buffer: 10mM Tris-HCl, pH 8.5) was 50-100 $\mu$ l.

In the extraction of pGETS103-ctb-cry j1 epi from *E. coli* DH5 $\alpha$  using Spin Miniprep Kit, the elution solution was preheated to 70 prior to the elution. In the extraction of pGETS103-ctb-cry j1 epi from *B. subtilis*, Hispeed Plasmid Midi Kit (Qiagen) was used with some modifications on the method: cells were pelleted from 400ml of LB culture (OD<sub>600</sub>: 0.8-1.2) by centrifugation at 3,000 $\times$ g for 15min at 4 and resuspended in 16ml of Buffer P1 containing 5mg/ml of lysozyme (from egg white, Wako); after incubation at 37 for 30min, 16ml of Buffer P2 were added and mixed gently but thoroughly, then incubated at room temperature for 5min; pre-chilled Buffer P3 was added and mixed well, then incubated on ice for 15min followed by centrifugation at 20,000 $\times$ g for 30min at 4 ; centrifuged again for 15min; the supernatant was applied to QIAGEN-tip equilibrated with 4ml of Buffer QBT; QIAGEN-tip was washed twice with Buffer QC (10ml/each time); plasmid was eluted with 5ml of Buffer QF; plasmid was precipitated by adding 3.5ml of isopropanol at room temperature and centrifuging at 15,000 $\times$ g for 30min at 4 , followed by washing with 2ml of room-temperature 70% ethanol and centrifuging at 15,000 $\times$ g for 10min at 4 ; finally, the pellet was air-dried and redissolved in 100 $\mu$ l of the elution buffer.

The extracted plasmid of pGETS103-ctb-cry j1 epi was dialysed for 1h on the semipermeable membrane (Spectra/Por membrane, MWCO 6-8000) against deionized water under stirring, and subsequently concentrated to the original volume by centrifugal filter devices (Microcon YM-10, Millipore) before the electroporation.

## 2.2.8 Expression of antigen peptides

### 2.2.8.1 Expression and purification of antigen peptides in *E. coli*

A single positive colony of *E. coli* BL21 (DE3) transformed by pET28a-ctb-cry j1 epi or

pET28a-ctb-cry j2 epi was grown in 2ml of LB/kanamycin medium under shaking at 37 °C for about 6h. Culture was then induced with IPTG (Takara) at a final concentration of 1mM and grown overnight at 27, 32 and 37 °C, respectively. Cells were pelleted by centrifugation and resuspended in 0.5ml of 20mM Tris-HCl (pH 8.0). After SDS-PAGE analysis, it was found that in both cultures expressing CTB-Cry j1 epi and CTB-Cry j2 epi, larger quantities of protein production were achieved at the induction temperature of 27 °C.

LB/kanamycin medium (50ml) was inoculated with 2ml of 5h culture of *E. coli* BL21 (DE3) transformants and grown under shaking at 37 °C for about 8h. The culture was induced with IPTG, and continued to grow overnight at 27 °C. The purification was performed using QIAexpress Type IV Kit (Qiagen) under denaturing conditions. Bacteria (0.5g wet weight) were harvested by centrifugation and resuspended in 2.5ml of the lysis buffer. After lysis under stirring for 60 min at room temperature, the lysate of bacteria was centrifuged, and the obtained supernatant was incubated with 0.6 ml of 50% Ni-NTA resin under shaking for 60 min at room temperature. Then the lysate-resin mixture was applied into a column. The flow-through was collected and the column was washed twice with the wash buffer (2.5ml/time). The recombinant protein was eluted with the elution buffer I (four times, 0.3 ml/time) followed by the elution buffer II (four times, 0.3ml/time). Fractions from the same buffer were pooled and applied to SDS-PAGE for analysis. Fractions of the elution buffer I and the elution buffer II were dialyzed against the dialysis solutions I-IV for 1.5h at room temperature respectively, and then against the dialysis solution V at 4 °C overnight. Buffers used are listed in Table 2.5.

Protein yield was measured with BCA Protein Assay Reagent Kit (Pierce) using bovine serum albumin (BSA) as the standard.



Table 2.5 Buffers used for the purification of antigen peptides

Buffer	Composition	pH
Lysis buffer	100 mM NaH <sub>2</sub> PO <sub>4</sub> , 10 mM Tris and 6 M GuHCl	8.0
Wash buffer	100 mM NaH <sub>2</sub> PO <sub>4</sub> , 10 mM Tris and 8 M urea	6.3
Elution buffer I	100 mM NaH <sub>2</sub> PO <sub>4</sub> , 10 mM Tris and 8 M urea	5.9
Elution buffer II	100 mM NaH <sub>2</sub> PO <sub>4</sub> , 10 mM Tris and 8 M urea	4.5
Dialysis solution I	Tris-EDTA buffer, 8 M urea	8.0
Dialysis solution II	Tris-EDTA buffer, 4 M urea	8.0
Dialysis solution III	Tris-EDTA buffer, 2 M urea	8.0
Dialysis solution IV	Tris-EDTA buffer, 1 M urea	8.0
Dialysis solution V	Tris-EDTA buffer, 0M urea	8.0

#### 2.2.8.2 Expression and extraction of the antigen peptide in *B. subtilis*

A single positive colony of *B. subtilis* 20038 (*B. subtilis* BEST2131 transformed by pHASH120, negative control), *B. subtilis* BEST2131 transformed by pHASH120-ctb-cry j1 epi, *B. subtilis* 168 *trpC2* (negative control) or *B. subtilis* 168 *trpC2* transformed by pGETS103-ctb-cry j1 epi was grown in 5ml of LB medium supplemented with chloramphenicol under shaking at 37 °C overnight. Bacteria were pelleted by centrifugation. The extraction of proteins was performed by adding 50µl of 2×SDS-PAGE sample buffer and heating for 5min at 90 °C. The suspension was centrifuged and 15µl of supernatant were applied on SDS-PAGE, followed by western blotting to detect the expression of the antigen peptide.

#### 2.2.9 SDS-PAGE

Protein samples mixed with 2×SDS-PAGE sample buffer were heated for 5min at 90 °C and loaded on SDS-PAGE for separation. Running conditions were as follows: 100V for 30min followed by 200V for 40min. Proteins were visualized by the staining solution of Coomassie brilliant blue R-250.

#### 2.2.10 Western blotting

To test antigenicities of expressed antigen peptides, western blotting was performed. Proteins isolated on SDS-PAGE were transferred onto the PVDF transfer membrane (Amersham) by the semidry method. After blocked with 1% skim milk in phosphate buffered saline (PBS) for 1h at room temperature or overnight at 4 °C, the membrane was incubated with the primary antibody for 1h at room temperature. Rabbit anti-cholera toxin antibody (1:5000 dilution, Sigma) was used as the primary antibody for both CTB-Cry j1 epi and CTB-Cry j2 epi, and rabbit anti-Cry j1 antibody (1:400 dilution, Hayashibara) and rabbit anti-Cry j2 antibody (1:400 dilution, LSL) were used as primary antibodies for CTB-Cry j1 epi and CTB-Cry j2 epi, respectively. Then the incubation with the secondary antibody (horseradish peroxidase-conjugated donkey anti-rabbit IgG, 1:25000 dilution, Amersham) was performed for 1h at room temperature. After the blocking and each incubation step, the membrane should be washed fully with PBS containing 0.5% Tween-20, ensuring to eliminate nonspecific absorption of milk or antibody completely. Signal was detected by ECL detection reagents (Amersham). Commercial CTB (Sigma), commercial Cry j1 antigen (Hayashibara) or commercial Cry j2 antigen (Hayashibara) were used as the positive controls.

### 2.3 Results and discussion

#### 2.3.1 Selection of major T cell epitopes from Cry j1 and Cry j2

Major T cell epitopes from Cry j1 and Cry j2 were selected based on the features of epitopes summarized in Table 2.6, which contains available epitopes from Cry j1 and Cry j2 for the design of immunotherapeutics, MHC class II types of human (HLA) presenting the epitopes, the reactivity of Cry j1 or Cry j2-specific T cell line against the epitopes as well as the finally selected epitopes in the literature [14]. In the literature, the epitopes containing the cysteine residue at the end were modified by removing them and those containing the cysteine residue in the middle position were eliminated regardless of the strength of the epitopes, based on the reason that influences of the cysteine residue on the release of epitopes from an artificial

polypeptide remain unknown. In addition, epitopes with the reactive ratio of 50% and undetermined HLA type were also eliminated.

Table 2.6 Analysis for the epitope selection in the literature [14]

Allergen	Available epitope	HLA type	Positive response (Strong positive response)%	Selected peptide
Cry j1	16-30	DQ6	61 (45)	
	66-80	Not determined	50 (11)	
	81-95	Not determined	61 (17)	
	91-105	DQ6	50 (11)	
	106-120	DR51	72 (28)	108-120
	111-125	Not determined	55 (28)	
	212-224	DP5	72 (33)	211-225
	301-315	Not determined	56 (11)	
	316-330	Not determined	50 (22)	
Cry j2	77-89	DR51	61 (39)	75-89
	90-104	DP5	50 (28)	
	150-164	Not determined	56 (0)	
	195-209	DR53	67 (28)	191-209
	245-259	DR15	50 (17)	
	345-359	DP5	50 (22)	353-364
	355-369	DQ6	67 (28)	
	360-374	Not determined	61 (22)	

However, we have some different ideas in the selection of T cell epitopes from Cry j1 and Cry j2. In the study, the effect of the cysteine residue was not considered. All the epitopes with reactivity higher than 50% as well as the strong reactivity higher than 15% were selected. Because Cry j2-96-107 possesses very strong reactivity based on another literature [9], it was chosen in stead of Cry j2-90-104. Because Cry j2-245-259 is presented by DR15 that was the

sole type among the selected HLA class II molecules, it was used as an epitope in order to achieve sufficient efficacy in a large population of allergic patients in spite of the reactivity of 50%. Cry j2-345-374 is a very important region for the design of the antigen peptide, therefore it was also selected in the study. Selected peptides and epitopes included are summarized in Table 2.7. Because effects of the arrangement of the individual epitope in the linked peptide on the antigenicity have not been well defined, they will be fused to CTB according to the original amino acid sequence order in the allergen protein in this study.

Table 2.7 Selected peptides and epitopes included from Cry j1 and Cry j2 in the study

Cry j1		Cry j2	
Peptide	Epitopes included	Peptide	Epitopes included
Cry j1-16-30	1	Cry j2-77-89	1
Cry j1-81-95	1	Cry j2-96-107	1
Cry j1-106-125	2	Cry j2-191-209	1
Cry j1-212-224	1	Cry j2-245-259	1
		Cry j2-345-374	3

### 2.3.2 Construction of fusion genes

The forward primer used for the construction of fusion genes, was designed according to the beginning coding sequence of CTB gene. The reverse primer consists of a complementary part with the template and an overhang of epitope gene. PCR was employed to add the epitope gene to the template stepwise, resulting in the final product with five Cry j1 epitope genes or seven Cry j2 epitope genes linked to CTB gene, respectively.

Sizes of resultant PCR products visualized on the agarose gel (Figure 2.5) are in good agreement with the calculated ones shown in Table 2.8, suggesting that epitope genes from Cry j1 or Cry j2 were successfully linked to CTB gene by this method. Moreover, results of sequencing analysis showed that sequences were identical to the designed ones and had correct orientations in expression plasmids. Nucleotide sequences and amino acid sequences of

CTB-Cry j1 epi and CTB-Cry j2 epi are shown in Figure 2.6. The final products were cloned into the cloning plasmid of pUC18, and then the expression plasmid of pET28a.

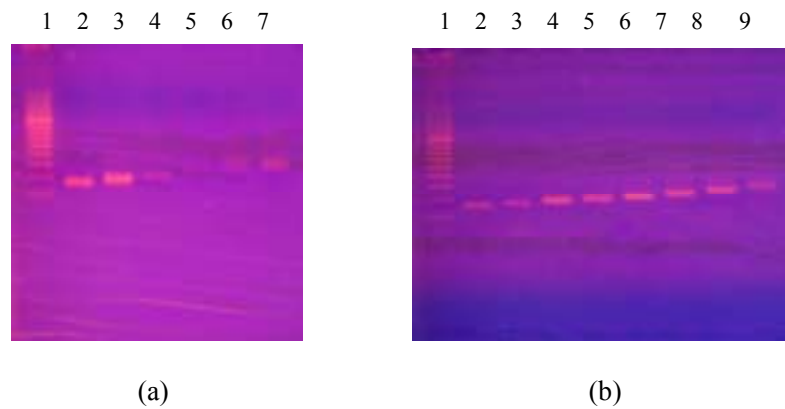


Figure 2.5 Electrophoretic analysis of products acquired from stepwise PCR

(a) PCR products in the linkage of CTB-Cry j1 epi gene. Lane 1, 200 bp marker; Lane 2, CTB gene; Lane 3, CTB-P1 gene; Lane 4, CTB-P1-P2 gene; Lane 5, CTB-P1-P2-P3 gene; Lane 6, CTB-P1-P2-P3-P4 gene; Lane 7, CTB-P1-P2-P3-P4-P5 gene (final product). (b) PCR products in the linkage of CTB-Cry j2 epi gene. Lane 1, 200bp marker; Lane 2, CTB gene; Lane 3, CTB-P'1 gene; Lane 4, CTB-P'1-P'2 gene; Lane 5, CTB-P'1-P'2-P'3 gene; Lane 6, CTB-P'1-P'2-P'3-P'4 gene; Lane 7, CTB-P'1-P'2-P'3-P'4-P'5 gene; Lane 8, CTB-P'1-P'2-P'3-P'4-P'5-P'6 gene; Lane 9, CTB-P'1-P'2-P'3-P'4-P'5-P'6-P'7 gene (final product).

Table 2.8 Calculated sizes of stepwise PCR products

Linkage of Cry j1 epitope genes to CTB gene			Linkage of Cry j2 epitope genes to CTB gene		
Reaction order	Primer name	Product size (bp)	Reaction order	Primer name	Product size (bp)
1	P1	354	1	P'1	348
2	P2	399	2	P'2	384
3	P3	429	3	P'3	414
4	P4	459	4	P'4	441
5	P5	498	5	P'5	486
			6	P'6	531
			7	P'7	576

(a)

DNA sequence of CTB-Cry j1 epi

ACACCTCAAAATATTACTGATTTGTGTGCAGAATACCACAACACACAAATACATACGCTAAATGATAAGAT  
ATTTTCGTATACAGAATCTCTAGCTGGAAAAAGAGAGATGGCTATCATTACTTTTAAGAATGGTGCAACTT  
TTCAAGTAGAAGTACCAGGTAGTCAACATATAGATTACAAAAAAAAGCGATTGAAAGGATGAAGGATA  
CCCTGAGGATTGCATATCTTACTGAAGCTAAAGTCGAAAAGTTATGTGTATGGAATAATAAACGCCTCAT  
GCGATTGCCGCAATTAGTATGGCAAAT-CAGAACCGTATGAAACTGGCGGATTGCGCGGTGGGCTTCGGC  
AGC-AAAATGCCGATGTATATTGCGGGCTATAAACGTTTCGATGGCCGT-CCGTGCGTGTTCATTAAACGT  
GTGAGCAACGTGATTATTCATGGCCTGCATCTGTATGGC-AGCATGAAAGTGACGGTGGCGTTCAACCAG  
TTCGGCCCCG

Amino acid sequence of CTB-Cry j1 epi

TPQNITDLCAEYHNTQIHTLNDKIFSYESLAGKREMAITFKNGATFQVEVPGSQHIDSQKKAIERMKDTRLI  
AYLTEAKVEKLCVWNNKTPHAIAAISMAN-QNRMKLADCAVGFGS-KMPMYIAGYKTFDGR-PCVFIKRV  
NVIIHGLHLYG-SMKVTVAFNQFGP

(b)

DNA sequence of CTB-Cry j2 epi gene

ACACCTCAAAATATTACTGATTTGTGTGCAGAATACCACAACACACAAATACATACGCTAAATGATAAGAT  
ATTTTCGTATACAGAATCTCTAGCTGGAAAAAGAGAGATGGCTATCATTACTTTTAAGAATGGTGCAACTT  
TTCAAGTAGAAGTACCAGGTAGTCAACATATAGATTACAAAAAAAAGCGATTGAAAGGATGAAGGATA  
CCCTGAGGATTGCATATCTTACTGAAGCTAAAGTCGAAAAGTTATGTGTATGGAATAATAAACGCCTCAT  
GCGATTGCCGCAATTAGTATGGCAAAT-GGCATTATTGCGGCGTATCAGAACCCGGCGAGCTGGAAA-CA  
GTTTCGCGAAACTGACGGGCTTCACGCTGATGGGC-ATTGATATTTTCGCGAGCAAAAACTTCCATCTGCA  
GAAAAACACGATTGGCACGGGC-AGCCGTGCGGAAGTGAGCTATGTGCATGTGAACGGCGCGAAATTC-  
TGCAAAGATATTAACTGAGCGATATTAGCCTGAAACTGACGAGCGGCAAAATTGCGAGCTGCCTGAAC  
GATAACGCGAACGGCTATTTTC

Amino acid sequence of CTB-Cry j2 epi

TPQNITDLCAEYHNTQIHTLNDKIFSYESLAGKREMAITFKNGATFQVEVPGSQHIDSQKKAIERMKDTRLI

AYLTEAKVEKLCVWNNKTPHAIAAISMAN-GIIAAYQNPASWK-QFAKLTGFTLMG-IDIFASKNFHLQKNTI  
GTG-SRAEVSIVHVNGAKF-CKDIKLSDISLKLTSGKIASCLNDNANGYF

Figure 2.6 Nucleotide sequences and amino acid sequences of fusion proteins

(a) Sequences of DNA and amino acids of CTB-Cry j1 epi. The order of linkage is CTB-Cry j1-16-30-Cry j1-81-95-Cry j1-106-125-Cry j1-212-224. (b) Sequences of DNA and amino acids of CTB-Cry j2 epi. The order of linkage is CTB-Cry j2-77-89-Cry j2-96-107-Cry j2-191-209-Cry j2-245-259-Cry j2-345-374.

### 2.3.3 Expression and antigenicities of antigen peptides expressed in *E. coli*

#### 2.3.3.1 Purification of antigen peptides

High expression levels of recombinant antigen peptides in *E. coli* led to the formation of insoluble inclusion bodies, which can be solubilized with strong denaturants such as 8M urea or 6M guanidine hydrochloride. In the study, 6M guanidine hydrochloride, rather than 8M urea was proved an effective denaturant and applied in the purification of antigen peptides.

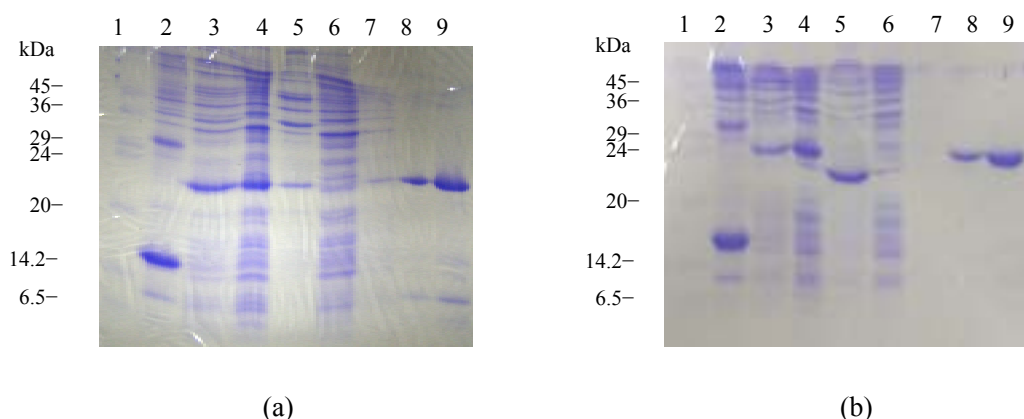


Figure 2.7 Purification of recombinant antigen peptides on the Ni-NTA resin affinity column  
(a) CTB-Cry j1 epi (b) CTB-Cry j2 epi. Lane 1, Molecular weight marker; Lane 2, Crude protein of CTB; Lane 3, Crude protein; Lane 4, lysate supernatant; Lane 5, Lysis debris; Lane 6, Flow-through; Lane 7, Washed fraction; Lane 8, Eluted fraction I; Lane 9, Eluted fraction II. Five  $\mu$ l protein samples were applied on SDS-PAGE.

A single band with high homogeneity was obtained from eluted fractions of the elution buffer I and II. Molecular mass of the antigen peptide visualized on SDS-PAGE (Figure 2.7) was in good agreement with the theoretical one: 23.7 kDa for CTB-Cry j1 epi or 26.3 kDa for CTB-Cry j2 epi.

Protein quantity measurement showed antigen peptides were efficiently expressed in *E. coli*, giving about 11 and 18 mg/L culture of purified CTB-Cry j1 epi and CTB-Cry j2 epi, respectively.

It was found that Cry j1 epi and Cry j2 epi could not be expressed without CTB fused to them, indicating that CTB also functions to stabilize short peptides expressed in microorganism-based systems (data not shown).

#### 2.3.3.2 Antigenicities of antigen peptides

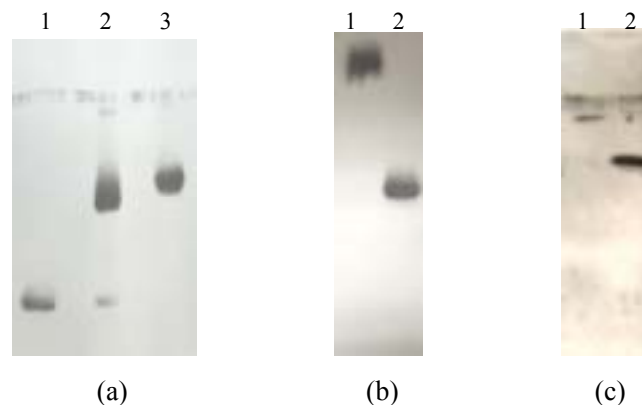


Figure 2.8 Antigenicities of purified antigen peptides

(a) The primary antibody used was anti-cholera toxin antibody. Lane 1, Commercial CTB; Lane 2, Purified CTB-Cry j1 epi; Lane 3, Purified CTB-Cry j2 epi. (b) The primary antibody used was anti-Cry j1 antibody. Lane 1, Commercial Cry j1 antigen; Lane 2, Purified CTB-Cry j1 epi. (c) The primary antibody used was anti-Cry j2 antibody. Lane 1, Commercial Cry j2 antigen; Lane 2, Purified CTB-Cry j2 epi.

One  $\mu$ l of commercial CTB ( $0.25\mu\text{g}/\mu\text{l}$ , MW 11kDa),  $1\mu\text{l}$  of commercial Cry j1 antigen ( $0.1\mu\text{g}/\mu\text{l}$ , MW 50kDa) or Cry j2 antigen ( $0.1\mu\text{g}/\mu\text{l}$ , MW 37kDa), and  $5\mu\text{l}$  of purified antigen peptides of CTB-Cry j1 epi ( $0.26\mu\text{g}/\mu\text{l}$ , MW 24kDa) or CTB-Cry j2 epi ( $0.31\mu\text{g}/\mu\text{l}$ , 27kDa) were analyzed.



To assess antigenicities of antigen peptides, western blotting was performed (Figure 2.8). The purified recombinant antigen peptides were proved antigenic against corresponding antibodies, i.e. CTB-Cry j1 epi was recognized by anti-cholera toxin and anti-Cry j1 antibodies; and CTB-Cry j2 epi was recognized by anti-cholera toxin and anti-Cry j2 antibodies.

In addition, anti-cholera toxin antibody did not recognize Cry j1 and Cry j2 antigens and anti-Cry j1 and anti-Cry j2 antibodies did not recognize CTB, and also anti-Cry j1 antibody did not recognize Cry j2 and vice versa (figure not shown). The result suggested no occurrence of cross-reactivity, consequently eliminating possibilities of false positive results.

#### 2.3.4 Insertion of the antigen gene in the genome of *B. subtilis* BEST2131

The *ctb-cry j1 epi* gene was cloned in the T-extended *EcoRV* ends of pHASH120. pHASH120 possesses two pBR322-derived antibiotic genes, the ampicillin-resistant gene and part of tetracycline-resistant ( $\Delta$ tet) gene that is not functional. pBR322-derived antibiotic genes are inert in *B. subtilis*. The vector also has a chloramphenicol-resistant gene and a blasticidin S-resistant gene, which are functional both in *E. coli* and in *B. subtilis*. pHASH120 is unable to self-replicate in *B. subtilis*.

*B. subtilis* BEST2131 has pBR322 sequences and an insertion of tetracycline-resistant gene between them in the *leuB* gene (*leuB*::pBRTc) of *B. subtilis* 168 *trpC2* genome. The tetracycline-resistant gene is only functional in *B. subtilis*.

The pBR322-derived sequences in the recombinant plasmid pHASH120 in the transformed BEST2131 can combine with the homologous sequences present in the BEST2131 genome, therefore hereafter the exchange of sequences in the plasmid and in the genome will happen naturally. The process is called homologous recombination. As a result, the genome obtained the *ctb-cry j1 epi* gene together with the promoter and RBS, which function to express the antigen peptide in *B. subtilis* genome. At the same time, BEST2131 with *ctb-cry j1 epi* inserted in the genome becomes sensitive to tetracycline and also blasticidin S, but acquires the ability against chloramphenicol. Figure 2.9 shows the schematic insertion process of the antigen gene in the genome of BEST2131 and the change in the antibiotic resistance before and after the insertion of the antigen gene.

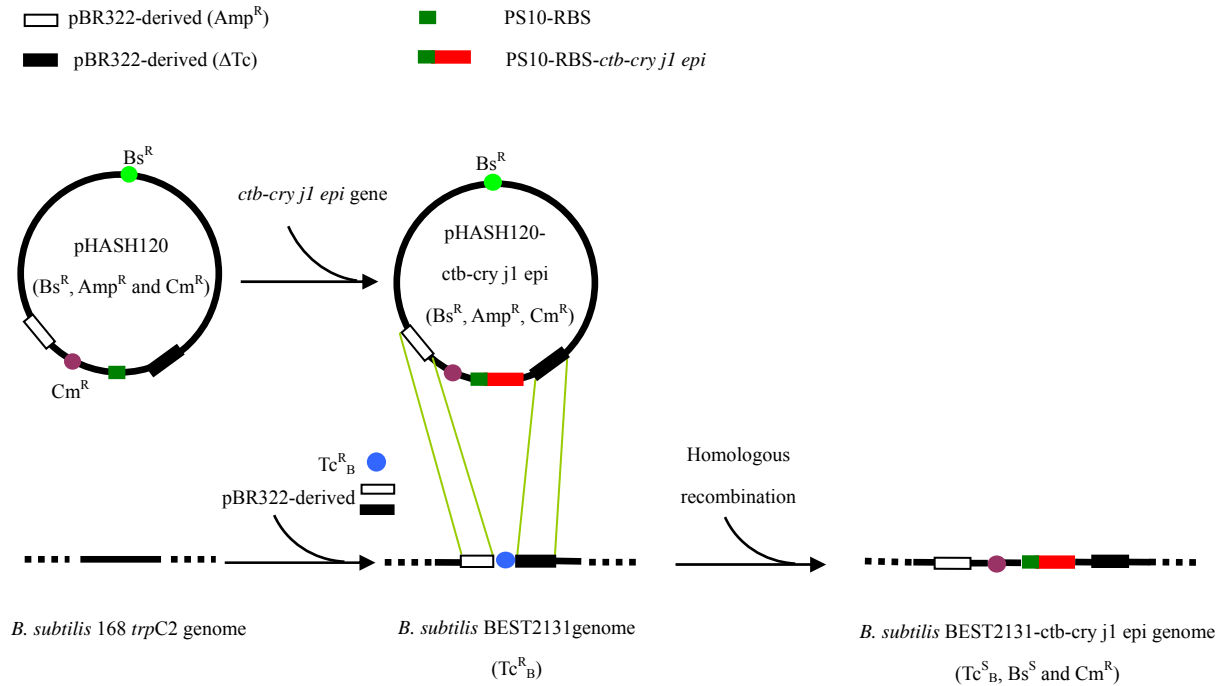


Figure 2.9 Schematic integration procedure of the antigen gene in the *B. subtilis* genome by homologous recombination via pBR322 sequences

Antibiotic-resistance genes indicated are: Amp, ampicillin; Cm, chloramphenicol;  $\Delta\text{Tc}$ , part of tetracycline-resistant gene;  $\text{Bs}$ , blasticidin S;  $\text{Tc}_B$ , tetracycline-resistant gene only functional in *B. subtilis*.  $^R$  and  $^S$  refer to “resistant” and “sensitive”, respectively.

### 2.3.5 Recovery of the antigen gene from the genome to a multi-copy plasmid

In order to test whether the antigen gene is functional or not, the vector of pGETS103 was used to recover the antigen gene from the genome.

The vector of pGETS103 is a multi-copy shuttle vector between *E. coli* and *B. subtilis*, the copy number was estimated to be 9 for *B. subtilis* and 20 for *E. coli*. The vector of pGETS103 consists of pTB522, a  $\theta$ -type replicating plasmid in *B. subtilis* from a thermophilic *Bacillus* strain and the full length of *E. coli* plasmid pBR322. Two antibiotic resistance genes are from pBR322 (ampicillin resistance and tetracycline resistance, which are only functional in *E. coli*), and one from pTB522 (tetracycline resistance, which is only functional in *B. subtilis*).

The vector of pGETS103 was linearized at the unique *Hind* III site prior to transforming *B.*

*subtilis* BEST2131-ctb-cry j1 epi. The linearized pGETS103 is unable to self-circularize, thus loses the ability of self-replication in *B. subtilis*. Circular pGETS103 can be established only after recovering the antigen gene from the genome of BEST2131-ctb-cry j1 epi by the homologous recombination. The circular plasmid then renders *B. subtilis* resistant to tetracycline. The process of recombinational transfer comprises four discrete steps: uptake of the linearized pGETS103, formation of homologous pairs, occurrence of recombination, and amplification of the plasmid [28]. Figure 2.10 shows the schematic process of extraction of the antigen gene from the genome to the plasmid.

It should be noted that the insertion of the antigen gene in the plasmid interferes with the downstream expression of pBR322-derived tetracycline resistant gene, resulting in the destruction of tetracycline-resistant activity in *E. coli*. But pTB522-derived tetracycline resistant gene was also functional in *B. subtilis*.

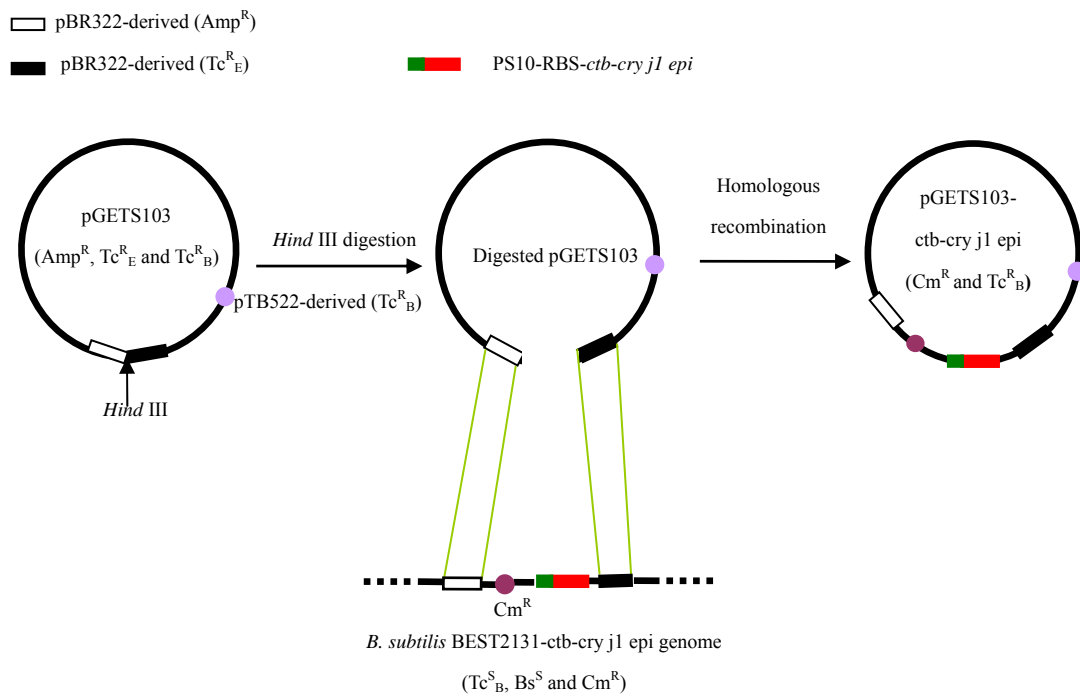


Figure 2.10 Schematic transfer procedure of the integrated antigen gene at the *B. subtilis* genomic pBR locus to a multi-copy plasmid by homologous recombination

Antibiotic-resistance genes indicated are: Amp, ampicillin;  $\text{Tc}_E$ , tetracycline-resistant gene only functional in *E. coli*;  $\text{Tc}_B$ , tetracycline-resistant gene only functional in *B. subtilis*; Cm, chloramphenicol; Bs, blasticidin S.  $^R$  and  $^S$  refer to “resistant” and “sensitive”, respectively.

### 2.3.6 Confirmation of insertion of *ctb-cry j1 epi* gene in plasmids and the genome

PCR products amplified from pHASH120-*ctb-cry j1 epi*, the BEST2131-*ctb-cry j1 epi* genome and pGETS103-*ctb-cry j1 epi* are shown in Figure 2.11.

Results suggested that we could obtain pHASH120-*ctb-cry j1 epi* in which the antigen gene was inserted in the desired orientation. This also guaranteed the correct insertion of the antigen gene in the genome. The insertion of the antigen gene in the genome and the recovery of the antigen gene from the genome to pGETS103 were also confirmed.

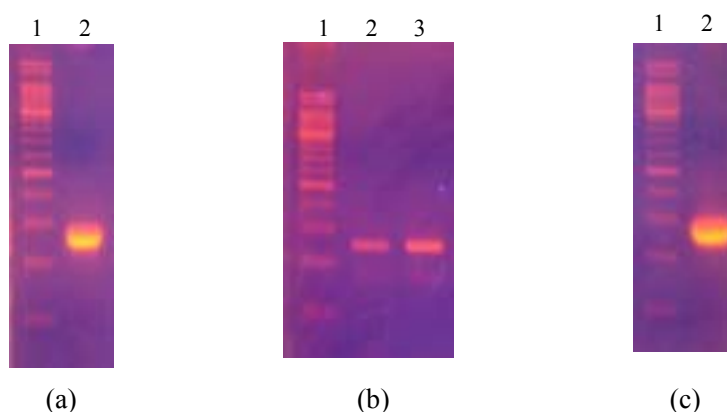


Figure 2.11 Electrophoretic analysis of PCR products to confirm the insertion of *ctb-cry j1 epi* (a) Confirmation of *ctb-cry j1 epi* in pHASH120-*ctb-cry j1 epi*. Lane 1, 200 bp marker; Lane 2, PCR product (b) Confirmation of *ctb-cry j1 epi* in the BEST2131-*ctb-cry j1 epi* genome. Lane 1, 200 bp marker; Lane 2 and 3, PCR products from colony No.1 and No. 2 (c) Confirmation of *ctb-cry j1 epi* in pGETS103-*ctb-cry j1 epi*. Lane 1, 200 bp marker; Lane 2, PCR product.

### 2.3.7 Expression of the antigen peptide in *B. subtilis*

The expression of CTB-Cry *j1 epi* in *B. subtilis* was examined by western blotting. CTB-Cry *j1 epi* was successfully expressed in *B. subtilis* when the gene was inserted in the plasmid, but little expression was observed when the antigen gene was integrated in the genome of *B. subtilis* (Figure 2.12). To test whether the antigen gene in the genome is functional or not, the inserted *ctb-cry j1 epi* gene in the genome was recovered by a multi-copy plasmid pGETS103, and CTB-Cry *j1 epi* was expressed in *B. subtilis 168 trpC2*. Results (Figure 2.12) indicated that

much quantity of CTB-Cry j1 epi was expressed in pGETS103. This indicated that the expression of the antigen gene was functional in the plasmid.

It should be noted that CTB-Cry j1 epi expressed in *E. coli* has larger molecular weight than that expressed in *B. subtilis*, which is due to the introduction of additional amino acids from the recombinant plasmid of pET28a to CTB-Cry j1 epi. The band below the CTB-Cry j1 epi (from pGETS103) expressed in *B. subtilis* 168 *trpC2* may be its degraded product.

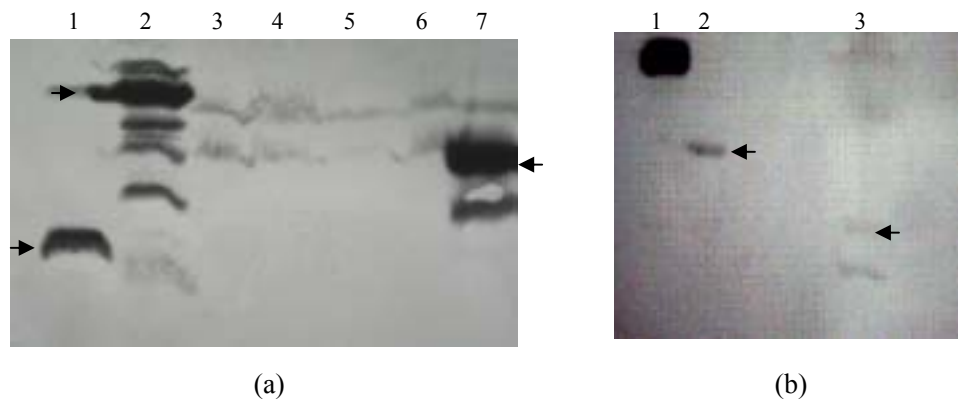


Figure 2.12 Expression of the *ctb-cry j1 epi* gene in *B. subtilis* (detected by western blotting) Experiments were performed according to the method described in Materials and methods 2.2.8.2. Briefly, 5ml of LB culture of *B. subtilis* was pelleted and 50 $\mu$ l of 2 $\times$ SDS-PAGE sample buffer was added to extract proteins by heating for 5min at 90 . (a) The primary antibody used was anti-cholera toxin antibody. Lane 1, Commercial CTB; Lane 2, *E. coli* expressing CTB-Cry j1 epi from pET28a; Lane 3, *B. subtilis* BEST20038 (BEST2131 transformed by pHASH120, negative control); Lane 4, *B. subtilis* BEST2131-CTB-Cry j1 epi, colony No.1; Lane 5, *B. subtilis* BEST2131-CTB-Cry j1 epi, colony No.2; Lane 6, *B. subtilis* 168 *trpC2* (negative control); Lane 7, *B. subtilis* 168 *trpC2* expressing CTB-Cry j1 epi from pGETS103. (b) The primary antibody used was anti-Cry j1 antibody. Lane 1, Commercial Cry j1 antigen; Lane 2, *E. coli* expressing CTB-Cry j1 epi from pET28a; Lane 3, *B. subtilis* 168 *trpC2* expressing CTB-Cry j1 epi from pGETS103.

Two  $\mu$ l of commercial CTB (0.25 $\mu$ g/ $\mu$ l, MW 11kDa), 3 $\mu$ l of commercial Cry j1 antigen (0.1 $\mu$ g/ $\mu$ l, MW 50kDa), 7.5 $\mu$ l of *E. coli* suspension expressing CTB-Cry j1 epi (MW 24kDa) from pET28a and 15 $\mu$ l of extraction from *B. subtilis* without or with CTB-Cry j1 epi (MW 19kDa) gene in the genome or in pGETS103 were analyzed. The arrow indicated the band of expressed CTB-Cry j1 epi.

It was assumed that the undetectable expression of the antigen peptide from the gene in the genome might be due to: (1) single copy number of the antigen gene in the genome; (2) some unknown factors in the genome which affected the normal expression of the inserted gene; (3) insufficient sensitivity and specificity of the methods to detect the antigen protein.

Middle wall protein (MWP) promoters from *Bacillus brevis* 47 are stronger promoters for the transcription of mRNA, therefore the stronger expression of proteins [30]. However, even if MWP promoters were used as the promoter of the antigen gene, no detectable amount of the antigen peptide from the gene in the genome was observed (data not shown). Complex factors in the genome for the expression of the foreign protein should be investigated.

## 2.4 Summary

Up to five major T cell epitopes from Cry j1 and seven from Cry j2 were selected on the basis of references in the study. To utilize the ability of CTB as the adjuvant and carrier, two genetically engineered fusion peptides, CTB-Cry j1 epi and CTB-Cry j2 epi, were designed. Genes encoding epitopes from Cry j1 or Cry j2 were fused to CTB gene in tandem by PCR. Results of sequencing analysis showed that sequences were identical to the designed ones and had correct orientations in expression plasmids.

Fusion genes were expressed firstly in *E. coli*. The expressed CTB-Cry j1 epi and CTB-Cry j2 epi were purified to a high homogeneity with Ni-NTA resin, giving about 11 and 18 mg/L culture of purified peptides, respectively. Results of antigenic reactivity by western blotting showed that recombinant CTB-Cry j1 epi or CTB-Cry j2 epi was recognized specifically by anti-cholera toxin as well as anti-Cry j1 (or anti-Cry j2) antibodies.

Natto is an ideal food carrier for the treatment of Japanese cedar pollinosis, because it is easily made and full of nutrients, and most importantly, is the favorite food of Japanese people. *B. subtilis* (natto) used for the fermentation of natto, is a closely related strain to *B. subtilis* 168 trpC2. The gene regulation of natto fermentation can function in *B. subtilis* 168 trpC2 if the relevant genes are appropriately transferred to corresponding loci of the genome by repeated transformation of *B. subtilis* 168 trpC2 with the genomic DNA from *B. subtilis* (natto). In the study, the *ctb-cry j1 epi* gene was inserted in the genome of *B. subtilis* BEST2131 (a strain with

pBR322 sequences in the *leuB* gene of 168 *trpC2*) by homologous recombination, which happened between two homologous DNA segments, i.e. pBR322-derived sequences, one in the BEST2131 genome and the other in the plasmid pHASH120 containing the antigen gene. The expression of CTB-Cry j1 epi from the gene in the genome of *B. subtilis* was not detected. To test whether the antigen gene is functional or not, the inserted *ctb-cry j1 epi* gene in the genome was recovered by a multi-copy plasmid pGETS103, and CTB-Cry j1 epi was expressed in *B. subtilis* 168 *trpC2*. Results indicated that much quantity of CTB-Cry j1 epi was expressed in pGETS103, suggesting the antigen gene is functional in plasmid. Complex factors in the genome for the expression of the foreign protein should be investigated.

## Chapter 3

### Utilization of CTB as a transporter:

### Expression of pentameric CTB and an attempt to assemble holotoxin-like complex in a cell-free system

#### 3.1 Introduction

The post-genomic era brings many challenges to the understanding of protein structure, function and interaction. Due to fast growth, easy handling and low cost, *E. coli* has long been the primary prokaryotic host for heterologous protein expression [31, 32]. However, the expression and accumulation of a foreign protein in *E. coli* often cause aggregates of the protein to form insoluble inclusion bodies [33, 34]. Sometimes the protein in these inclusion bodies can be refolded *in vitro* to produce functional protein [35, 36], but this is not always possible, and renaturation can be expensive and time-consuming. Moreover, many proteins, for example, structurally complex oligomeric proteins and those containing multiple disulfide bonds, are not easily recovered to an active conformation following chemical denaturation [34]. The expression of heterologous proteins in other bacterial systems also often encounters the same problems as those in *E. coli*. All these restrict the application of bacteria-based systems in the expression of structurally complex oligomeric proteins.

Since the advent of the first real cell-free protein expression system in the 1950s, the cell-free system has attracted considerable attention as an alternative protein synthesis method, especially with the appearance of highly productive, commercial kits, which are available for the convenient production of a broad variety of proteins. The cell-free system is an *in vitro* protein expression system. It contains all the necessary elements for the expression of proteins, such as ribosome, enzymes, translation factors, amino acids, tRNA, energy source supplied by ATP and GTP, buffers and salts such as Mg, and DNA or mRNA is used as the template. Two cell-free



protein expression systems are available: the prokaryotic system based on the *E. coli* lysate and the eukaryotic system based on the rabbit reticulocyte extract or the wheat germ embryo extract.

Continuous exchange cell-free (CECF) technology was proposed firstly by Spirin *et al* [37]. The schematic working principle of CECF is shown in Figure 3.1. This technology provides continuous removal of by-products and a steady supply of substrates necessary to sustain the reaction, resulting in high levels of protein synthesis. This is realized by employing a semi-permeable membrane (10kDa cut-off) to create two compartments in the reaction vessel: a reaction compartment in which transcription/translation occurs and the protein product is retained; a feeding compartment into which inhibitory reaction by-products diffuse and from which reaction substrates and energy components are continuously supplied for sustained synthesis.

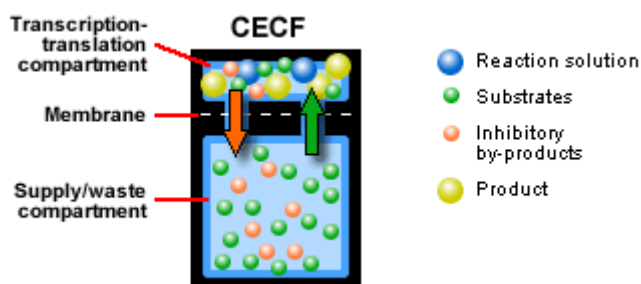


Figure 3.1 Continuous exchange cell-free (CECF) technology

(<https://www.roche-applied-science.com/sis/proteinexpression/index.jsp>)

The cell-free system provides powerful tools to synthesize any desired protein, including native proteins, proteins toxic to living cells and artificially modified proteins [37]. Moreover, the cell-free system has the strong ability in expressing soluble, properly folded proteins [38]. Most attractively, in contrast to bacterial expression systems, the cell-free system offers considerable flexibility to investigate the interaction of proteins by coexpressing them.

Fundamentals to the cell-free system are rational gene design, usage of linear or circular template with optional tag in N- or C- terminus for purification, and expression screening and scale up. The schematic experimental flowchart (take the *E. coli* cell-free system as an example) is shown in Figure 3.2. To use the system, the gene of interest must be positioned downstream

of a T7 promoter and between an optimized 5'- and 3'- UTR (untranslated region). The vector used for expression should include the following elements and structural features: target gene must be under control of T7 promoter that is located downstream from an RBS sequence; distance between T7 promoter and an initiating ATG triplet should not exceed 100 base pairs; distance between RBS sequence and the ATG should not be more than 5-8 base pairs; and T7 terminator sequence must be present at the 3' end of the gene. In theory, any vector with this elements and features can be used.

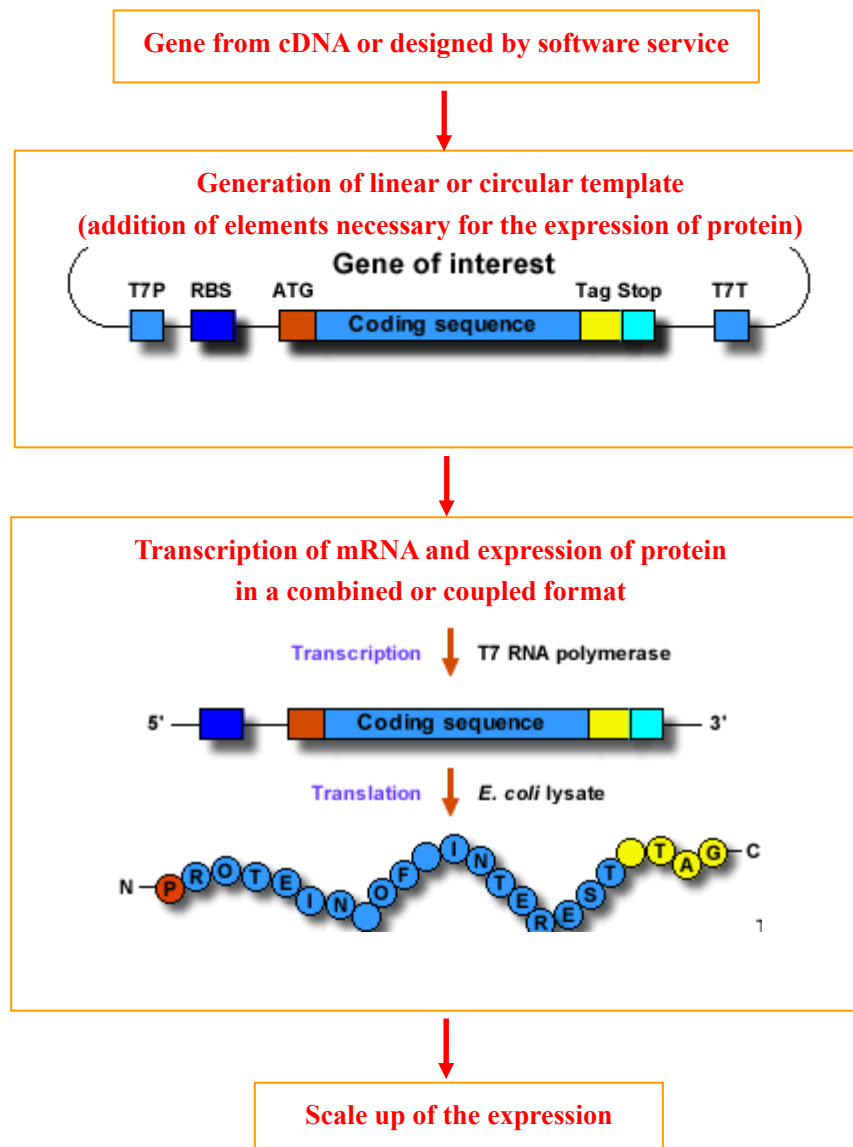


Figure 3.2 Combined or coupled *in vitro* transcription/translation in the cell-free system  
(Part of the figure quoted from RTS application manual for cell-free protein expression )

It was found that CTA interacted with CTB primarily through its A<sub>2</sub> domain, which inserted into the center of the ring formed by CTB pentamer, and CTA<sub>1</sub> was not required to maintain the stability of the noncovalent interactions between CTA<sub>2</sub> and B pentamer [39]. The interactions between CTA<sub>2</sub> and B pentamer that occur within the upper end of the pentamer pore are exclusively hydrophobic, while the interactions that occur in the lower regions of the pentamer core are largely hydrophilic [40]. It was also found that CTA would only associate with CTB that was in the process of assembling, i.e. the pathway of holotoxin assembly involved the association of CTA, not with the fully assembled CTB pentamer, but with CTB intermediate [41]. In addition, the disulfide bond formation (between the cysteine residues of Cys-9 and Cys-86 in CTB) is required to generate and stabilize correctly structured B monomers capable of forming pentamer [42].

A new strategy for the delivery of vaccines and antigens is to genetically engineer the interested peptide or protein linked to the nontoxic CTA<sub>2</sub> segment and coexpress CTB to create a holotoxin-like chimera for generating CT-based mucosal vaccines or antigens with reduced toxicity and high efficiency. However, to our knowledge, the recombinant CTB expressed in *E. coli* was in an insoluble form, which is the greatest obstacle for the assembly of holotoxin-like complex. The only *in vitro* assembly method involved denaturation and reactivation of CTA<sub>2</sub>-fusion protein and CTB, which was troublesome and actually difficult for the practical application [43].

RTS 100 *E. coli* Disulfide Kit (50μl/reaction) developed by Roche is a CECF system based on *E. coli* lysate for the expression of disulfide bonded proteins from plasmid templates. The kit uses the redox buffer to maintain the system under oxidizing conditions, the disulfide isomerase for the formation and the rearrangement of disulfide bonds, and increased amounts of GroE chaperones to guarantee the production of soluble, properly folded proteins. The kit can provide the platform to assemble the holotoxin-like complex by coexpressing CTB and the fusion protein of interested peptide or protein with CTA<sub>2</sub> simultaneously. However, before the assembly of the holotoxin-like complex is performed, the ability of CTB in the formation of pentamer has to be verified.

Assembling cholera toxin-like complex by coexpressing CTB and the fusion protein of green fluorescence protein (GFP) linked to CTA<sub>2</sub> in the cell-free system was attempted. GFP was

selected as a model protein because of no requirement of cofactors and substrates for fluorescence activity, allowing for direct, real-time detection.

### 3.2 Material and methods

#### 3.2.1 Bacterial strains, plasmids and media

*E. coli* strain JM109 (Takara) was used for the cloning of recombinant plasmid pET28a, and kanamycin (25µg/ml) was used as the selection antibiotic for the positive transformants. *E. coli* strain BL21 (DE3) (Novagen) was used for the expression of CTB. *E. coli* strain XL1 blue was used for the cloning of recombinant plasmid of pIVEX2.4d (Roche), and ampicillin (50µg/ml) was used as the selection antibiotic. pEGFP vector (Clontech), harboring the enhanced green fluorescent protein (EGFP) gene, was amplified in *E. coli* strain JM109 with the selection by ampicillin (100µg/ml). *E. coli* transformation method was according to Materials and methods 2.2.6.1 of chapter 1. All the *E. coli* strains were grown at 37 °C in LB medium supplemented with the appropriate antibiotic.

Kanamycin and ampicillin were purchased from Wako and Sigma, respectively.

#### 3.2.2 Construction of genes by PCR amplification

Genes of *ctb* with different structures and *gfp-cta<sub>2</sub>* fusion gene were constructed using the primers shown in Table 3.1. CT gene (PCR product from the genome of *Vibrio cholerae* strain 569B, kindly provided by Prof. Takeshi Honda of Osaka University) was applied as templates for the construction of *ctb* and *cta<sub>2</sub>* genes. In the case of the construction of *ctb* genes, primers of *Bam*HI-CTB-FW and CTB-RV-*Hind* III-2 were used to introduce the *Bam*HI site to 5' and the *Hind* III site to 3' of *ctb* gene, and after cloned in pET28a, the expressed CTB had an N-terminal His×6 tag followed by an extra segment of 22 amino acids which was introduced by the recombinant plasmid pET28a; primers of *Nco*I-CTB-FW and CTB-*Bam*HI-RV were used to introduce *Nco*I and *Bam*HI sites, the resultant CTB having the structure nearly the same with the natural one, was obtained after cloned in pET28a; forward primers of His×3-CTB-FW and

*Nco*I-His×6-CTB-FW, and the reverse primer of CTB-*Bam*HI-RV were used to introduce a His×6 tag which was directly attached to N-terminus of CTB; primers of *Nco*I-CTB-FW and CTB-*Xho*I-RV were used for the introduction of a His×6 tag on the C-terminus of CTB after the gene was inserted in pET28a. So four kinds of *ctb* genes, which are summarized in Table 3.1, were constructed to test what had affected the pentamer formation: His×6 tag, the extra segment of amino acids or expression system itself. In the case of the construction of *gfp-cta<sub>2</sub>* fusion gene, *cta<sub>2</sub>* was amplified with the primer GFP-CTA<sub>2</sub>-FW to insert part of *gfp* sequence. The inserted part of *gfp* sequence is convenient for the linkage with *gfp* gene to obtain the fusion gene of *gfp-cta<sub>2</sub>*. The *gfp* gene was amplified using pEGFP vector as the template.

Table 3.1 Primers and templates used for the construction of *ctb* and *gfp-a<sub>2</sub>*

Purpose	Template	Type	Name	Sequence* (5'-3')
Construction of <i>ctb</i> genes	CT gene	Forward	<i>Bam</i> HI-CTB-FW	actgc <b>ggatc</b> cacacctcaaaatattactg
			<i>Nco</i> I-CTB-FW	cggccatgggtcctcaaaatattactgatt
			His×3-CTB-FW	catcatcacacacctcaaaatattactgat
			<i>Nco</i> I-His×6-CTB-FW	cggccatgggtcaccaccaccatcatcacacct
		Reverse	CTB-RV- <i>Hind</i> III-2	tggagcaagctt <b>ta</b> atttgccataactaatt
			CTB- <i>Bam</i> HI-RV	gcc <b>ggatc</b> <b>ctta</b> atttgccataactaattgc
			CTB- <i>Xho</i> I-RV	cggctc <b>gag</b> atttgccataactaattgcggc
Construction of genes of <i>gfp</i> , <i>cta<sub>2</sub></i> and <i>gfp-cta<sub>2</sub></i>	pEGFP vector	Forward	<i>Nco</i> I-GFP-FW	gcgccatgggtgagcaagggc
		Reverse	GFP-RV	ctgtacagctgtccatgc
	CT gene	Forward	GFP-CTA <sub>2</sub> -FW	<u>gcatggacgagctgtacaagagta</u> ataacttgcgataaaa
		Reverse	<i>Bam</i> HI-CTA <sub>2</sub> -RV	cgc <b>ggatc</b> ctcataattcatcctaattct

\*Sequences indicated in bold are sites for restriction enzymes, red nucleotides represent the stop codon, and the underlined sequence is the part of *gfp* gene for the construction of *gfp-cta<sub>2</sub>* fusion gene.

Conditions of PCR reaction for the amplification of genes of *ctb*, *cta<sub>2</sub>* and *gfp* were as follows: 98 °C, 5min preheating, followed by 25 cycles of denaturing (98 °C, 15sec), annealing (65 °C, 2sec) and elongation (74 °C, 30sec). For the ligation of *gfp* and *cta<sub>2</sub>* genes, PCR reaction

conditions were slightly different: PCR products from *gfp* and *cta<sub>2</sub>* mixed with reaction reagents without primers were preheated at 98 °C for 5min, followed by 10 cycles of denaturing (98 °C, 15sec), annealing (65 °C, 15sec) and elongation (74 °C, 30sec). Then, after the addition of the primers of *Nco*I-GFP-FW and *Bam*HI-CTA<sub>2</sub>-RV, 25 cycles of denaturing (98 °C, 15sec), annealing (65 °C, 2sec) and elongation (74 °C, 30sec) were performed.

All primers were purchased from Proligo. PCR was accomplished with high fidelity KOD DNA polymerase (Toyobo). After each step of PCR, the product was purified using PCR Purification Kit (Qiagen) or purified from the agarose gel (Takara) using Gel Extraction Kit (Qiagen). PCR products were analyzed on the 2% agarose gel stained with ethidium bromide by electrophoresis.

### 3.2.3 Construction of recombinant plasmids

Structure and cloning/expression region of *E. coli* plasmid pET28a is shown in Figure 2.1 of Chapter 2, and that of pIVEX2.4d is shown in Figure 3.3. The pIVEX2.4d vector contains all regulatory elements necessary for protein expression in RTS *E. coli* cell-free systems, which are based on a combination of T7 RNA polymerase and prokaryotic cell lysates. The vector introduces an N-terminal His×6 tag, which allows one-step purification of the protein by Ni-NTA affinity chromatography. Moreover, in most cases, His×6 tag (only 0.84kDa) does not interfere with the structure of function of the purified protein for a wide variety of proteins.

In the case of cloning and expression of the *ctb* gene, the resultant DNA fragments from PCR were digested with corresponding restriction enzymes (all purchased from Takara) and cloned into pET28a plasmid. After amplified in *E. coli* JM109, the plasmids were used for the expression in the RTS cell-free system or for the transformation in *E. coli* BL21 (DE3) to obtain expressed CTB from *E. coli*. Restriction sites of *ctb* gene, and structures and characteristics of expressed CTB are shown in Table 3.2.

In the case of cloning and expression of the *gfp-cta<sub>2</sub>* gene, the resultant DNA fragments from PCR were digested with restriction enzymes of *Nco*I and *Bam*HI. After the digested gene was cloned into corresponding sites of pET28a, no expression of protein was found. Therefore, the recommended vector of pIVEX2.4d by the RTS *E.coli* cell-free system was used for expression.

After amplification in *E. coli* XL1 blue, the recombinant plasmid of pIVEX2.4d was used for the expression in the cell-free system.

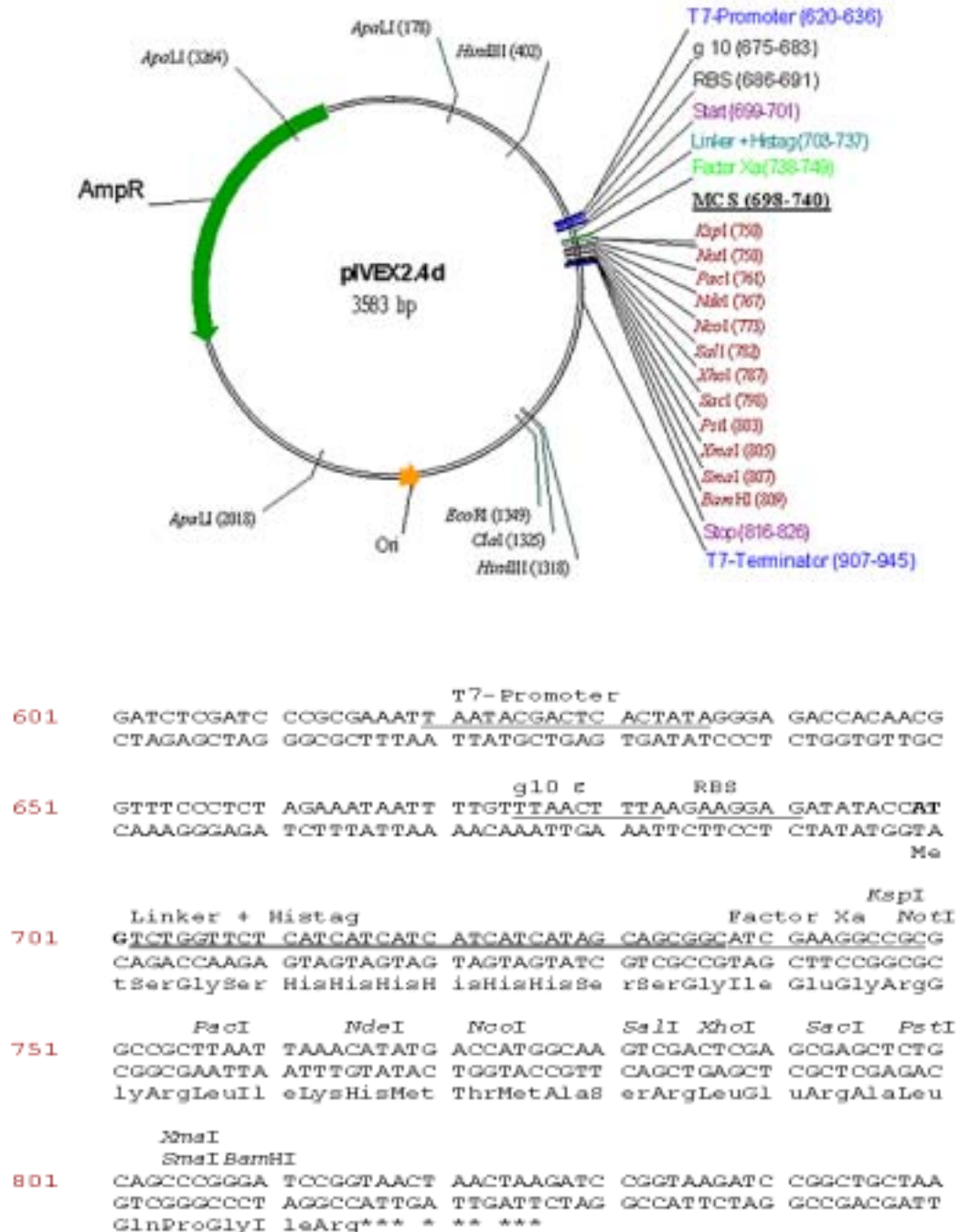


Figure 3.3 Structure and cloning/expression region of pIVEX2.4d for the RTS cell-free system

(<https://www.roche-applied-science.com/sis/proteinexpression/index.jsp>)

Table 3.2 Restriction site of *ctb* gene, and structure and characteristics of expressed CTB

Restriction site of <i>ctb</i>	Structure of CTB (N→C)	Characteristics of CTB
<i>Bam</i> HI/ <i>Hind</i> III	His×6-22aa-CTB	With an N-terminal His×6 tag followed by an additional segment of 22 amino acids introduced by the recombinant plasmid pET28a
<i>Nco</i> I/ <i>Bam</i> HI	CTB	Nearly the same with the natural CTB
<i>Nco</i> I/ <i>Bam</i> HI	His×6-CTB	With an N-terminal His×6 tag
<i>Nco</i> I/ <i>Xho</i> I	CTB-His×6	With a C-terminal His×6 tag

All the enzymatic digestion products were purified from the agarose gel using Gel Extraction Kit, and recombinant plasmids were extracted using Spin Miniprep Kit (Qiagen). Sequences and the orientations of inserted genes in recombinant plasmids were analyzed using the method described in Materials and methods 2.2.6 of Chapter 2.

### 3.2.4 Protein expression

#### 3.2.4.1 Expression of CTB in *E. coli*

CTB (from all of recombinant plasmids of pET28a constructed in the study) was expressed in 2ml of LB/kanamycin culture under shaking at 37 °C with the induction of IPTG as described in Method and materials 2.2.8.1 of Chapter 2. The cells were pelleted by centrifugation, resuspended in 0.5ml 20mM Tris-HCl (pH 8.0) and used for SDS-PAGE analysis.

#### 3.2.4.2 Optimal expression time in the RTS cell-free system

Expression of CTB (from pET28a-*Nco*I-*ctb*-*Bam*HI) and GFP-CTA<sub>2</sub> in the RTS cell-free system was performed according to the instruction of RTS 100 *E. coli* Disulfide Kit (Roche). Preparation of working solutions is briefly described in Table 3.3. The recombinant plasmid



(0.5µg in 5µl water) was used as the template for the expression of protein. After rinsed with DNase- and RNase-free water, the feeding compartment in the CECF device was filled with 1ml of feeding solution, and the reaction compartment with 50µl of reaction solution. All the compartments of the device were covered carefully with the adhesive film, then the device was inserted into the plastic frame supplied with the kit. The reaction was started by placing the frame into the plate shaker (Nissin) setted at 680rpm in the 30 °C incubator. The reaction was performed at the different time of 4, 8, 12, 24 or 36h to investigate the optimal expression of proteins. The CECF device and the plate shaker are shown in Figure 3.4.

The expressed CTB was visualized by western blotting using the method described in Materials and method 2.2.10 of Chapter 2. The expression of GFP-CTA<sub>2</sub> was visualized by fluorescence, measured using an ultraviolet transilluminator (Funakoshi) at an excitation wavelength of 365nm.

Table 3.3 Preparation of working solutions for protein expression in the RTS cell-free system

Solution	Component and preparation method (one 50µl reaction )
Reaction solution	1. Activated lysate: mix 25µl <i>E. coli</i> lysate and 1µl lysate activator carefully and incubate at room temperature for 10-20min 2. Reaction solution: 7µl reaction mixture, 7µl amino acid mixture, 1µl methionine and 25µl activated lysate, 5µl water and 0.5µg of circular DNA template in 5µl water or TE buffer
Feeding solution	640µl feeding mixture, 140µl amino acid mixture, 20µl methionine and 200µl water

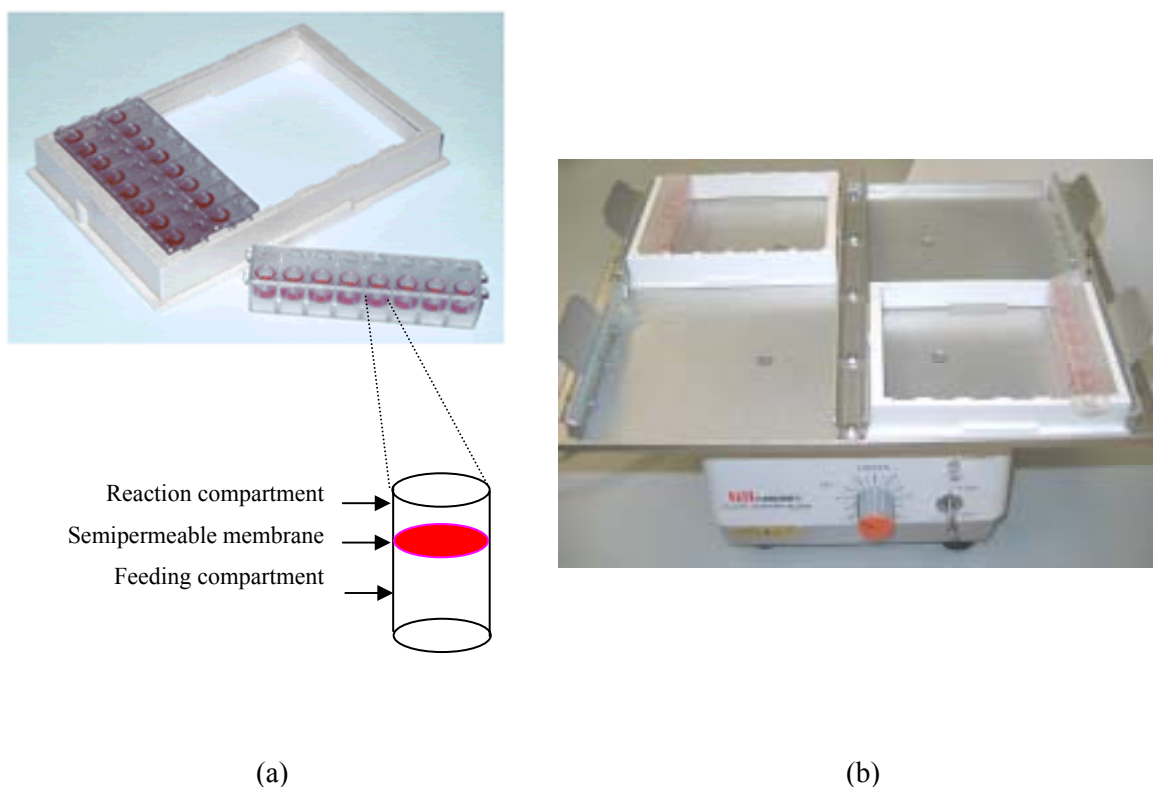


Figure 3.4 CECF device and plate shaker for the expression of protein in the RTS cell-free system

(a) CECF device (b) plate shaker

### 3.2.4.3 Expression of proteins in the RTS cell-free system

Expression of CTB (from all of recombinant plasmids constructed in the study), GFP-CTA<sub>2</sub>, and coexpression of GFP-CTA<sub>2</sub> and CTB (from pET28a-*Nco*I-ctb-*Bam*HI) were performed according to the method described above with the reaction time of 24h. The plasmid pIVEX2.3-GFP WT (wild type GFP) supplied by the kit was expressed as the positive control at the same time.

### 3.2.5 Investigation of the formation of CTB pentamer

#### 3.2.5.1 Western blotting

To visualize the pentameric CTB, protein samples were treated before applied on SDS-PAGE using the method different from that described in Materials and methods 2.2.9 of Chapter 2. No mercaptoethanol (a reductant for the disulfide bond) was added in 2×sample buffer, and samples were not treated with heat. The preparation method of SDS-PAGE, buffer compositions and electrophoretic conditions were the same with those described before.

After the electrophoresis, the visualization of pentameric CTB was performed by western blotting using the method described before, except that only the anti-cholera toxin antibody was used as the primary antibody for the detection.

### 3.2.5.2 GM<sub>1</sub>-ganglioside binding assay

GM<sub>1</sub>-ELISA was performed to detect the GM<sub>1</sub>-ganglioside binding activity of CTB synthesized in the RTS cell-free system. The 96-well microtiter plate (Nunc, maxisoap type) was coated with GM<sub>1</sub>-monosialoganglioside (Sigma) by incubating the plate with 50μl/well of GM<sub>1</sub> methanol solution (0.2μg/ml) in vacuum desiccator at room temperature overnight. The plate was blocked by incubation in the blocking buffer (1% BSA/PBS, 100μl/well) for 30min at 25 °C, and the blocking buffer was removed by flicking the plate on the absorbant paper. Serial dilutions (50, 25, 12.5, 6.25 and 3.125ng/well) of commercial CTB, CTB (from pET28a-*NcoI*-ctb-*Bam*HI and pET28a-*NcoI*-ctb-*Xho*I-His×6) expressed in the RTS cell-free system (500-, 1000- and 2000-times diluted) and negative control (only buffer) were loaded in the wells (50μl/well) and incubated for 30min at 25 °C. Subsequently, the plate was incubated in the rabbit anti-cholera toxin primary antibody (Sigma) in the washing buffer (1:5000 dilution, 50μl/well) at 25 °C for 45min, followed by incubation with the anti-rabbit IgG-HRP secondary antibody (Amersham) in washing buffer (1:5000 dilution, 50μl/well) for 30min at 25 °C. After each incubation step, the plate was washed fully (three or four times) with the washing buffer (200μl/well), ensuring complete elimination of nonspecific absorption of antibody. Finally, the chromogenic substrate of ABTS (2,2'-Azino-Bis (3-ethylbenzthiazoline-6-sulfonic Acid)) (Sigma) (50μl/well) was added and the plate was incubated at 25 °C for 5, 10, 15, 20, 30 and 40min, respectively, to develop the color. Absorbances were measured in the microplate reader at 405nm.

Linear standard curve of absorbances versus the known concentrations of commercial CTB, was established. The expression levels of pentameric CTB in the RTS cell-free system were estimated by comparing the absorbances with the standard curve.

### 3.2.6 Investigation of the formation of holotoxin-like complex of GFP-CTA<sub>2</sub> and CTB

#### 3.2.6.1 Purification of coexpressed GFP-CTA<sub>2</sub> and CTB

To investigate the formation of holotoxin-like complex of GFP-CTA<sub>2</sub> and CTB, purification of coexpressed GFP-CTA<sub>2</sub> and CTB was performed. Because GFP-CTA<sub>2</sub> has a His × 6 tag on its N terminus, CTB without His × 6 tag (from pET28a-*NcoI*-ctb-*Bam*HI) will be purified together if the complex of GFP-CTA<sub>2</sub> and CTB forms. The purification of mixture of GFP-CTA<sub>2</sub> and CTB was also performed as the control.

The purification was done according to the instruction of QIAexpress Type IV Kit (Qiagen) under native conditions with some modifications. Buffers used in the purification are listed in Table 3.4. The pooled solution from four reactions (about 200μl) was mixed with 800μl of the lysis buffer to give a 1ml solution. After incubated with 0.25ml of 50% Ni-NTA resin by gentle shaking at 4 °C for 60min, the mixture was loaded into a column to collect the flow-through. The resin was washed twice with the wash buffer (1ml/time), followed by elution with the elution buffer I (0.125ml/time, four times), the elution buffer II (0.125ml/time, four times) and the elution buffer III (0.125ml/time, four times), respectively.

Samples were concentrated to the appropriate volume by centrifugal filter devices (Amicon Ultra-4, 5000 MWCO, Millipore) and detected by western blotting using anti-cholera toxin antibody as the primary antibody.

Table 3.4 Buffers used for the purification of proteins expressed in the RTS cell-free system

Buffer	Composition	pH
Lysis buffer	50mM NaH <sub>2</sub> PO <sub>4</sub> , 300mM NaCl	8
Wash buffer	50mM NaH <sub>2</sub> PO <sub>4</sub> , 300mM NaCl, 5mM imidazole	8
Elution buffer I	50mM NaH <sub>2</sub> PO <sub>4</sub> , 300mM NaCl, 20mM imidazole	8
Elution buffer II	50mM NaH <sub>2</sub> PO <sub>4</sub> , 300mM NaCl, 100mM imidazole	8
Elution buffer III	50mM NaH <sub>2</sub> PO <sub>4</sub> , 300mM NaCl, 250mM imidazole	8

### 3.2.6.2 SDS-PAGE

To visualize the fluorescent GFP-CTA<sub>2</sub>, and if possible, the complex of GFP-CTA<sub>2</sub> and pentameric CTB on SDS-PAGE, protein samples were treated using the method as described above: no mercaptoethanol was added in 2×sample buffer, and samples were not treated with heat. The preparation method of SDS-PAGE, buffer compositions and electrophoretic conditions were also the same with those described before. After the electrophoresis, the fluorescence was visualized by putting the gel in an ultraviolet transilluminator with an excitation wavelength of 365nm.

### 3.2.6.3 Western blotting

To understand the actual molecular weight of GFP-CTA<sub>2</sub>, protein samples were treated with heat and mercaptoethanol before loaded on SDS-PAGE. Western blotting was performed according to the method described before, except that anti-rabbit polyclonal GFP antiserum (Invitrogen) was used as the primary antibody.

To investigate the formation of complex of GFP-CTA<sub>2</sub> and CTB, samples in the purification were treated with heat and mercaptoethanol and applied for western blotting detection. Anti-cholera toxin antibody was used as the primary antibody.

## 3.3 Results and discussion

### 3.3.1 Construction of genes and recombinant plasmids

Sizes of resultant PCR products (*Bam*HI-*ctb*-*Hind* III not shown) visualized on the agarose gel (Figure 3.5) are in good agreement with the calculated ones shown in Table 3.5.

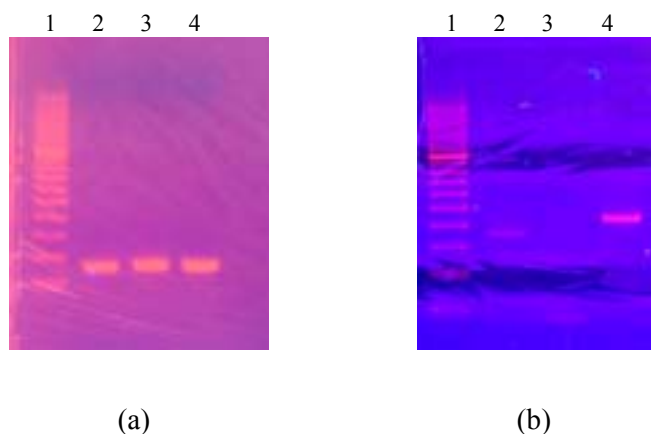


Figure 3.5 Electrophoretic analysis of PCR products

(a) PCR products for the construction of *ctb* genes. Lane 1, 200 bp marker; Lane 2, *Nco*I-*ctb*-*Bam*HI; Lane 3, *Nco*I-*his*×6-*ctb*-*Bam*HI; Lane 4, *Nco*I-*ctb*-*Xho*I-*his*×6. (b) PCR products for the construction of GFP-CTA<sub>2</sub> gene. Lane 1, 200bp marker; Lane 2, *Nco*I-*gfp*; Lane 3, *gfp*\*-*cta*<sub>2</sub>-*Bam*HI (\*part of *gfp* gene (20bp)); Lane 4, *Nco*I-*gfp*-*cta*<sub>2</sub>-*Bam*HI.

Table 3.5 Calculated sizes of PCR products

PCR product	Size (bp)
<i>Bam</i> HI- <i>ctb</i> - <i>Hind</i> III	332
<i>Nco</i> I- <i>ctb</i> - <i>Bam</i> HI	329
<i>Nco</i> I- <i>his</i> ×6- <i>ctb</i> - <i>Bam</i> HI	347
<i>Nco</i> I- <i>ctb</i> - <i>Xho</i> I- <i>his</i> ×6	327
<i>Nco</i> I- <i>gfp</i>	726
<i>gfp</i> *- <i>cta</i> <sub>2</sub> - <i>Bam</i> HI	161
<i>Nco</i> I- <i>gfp</i> - <i>cta</i> <sub>2</sub> - <i>Bam</i> HI	861

\*part of *gfp* gene (20bp).

PCR products were cloned into the expression plasmid of pET28a or pIVEX2.4d. Results of sequencing analysis showed that sequences were identical to the designed ones and had correct orientations in expression plasmids.

Sequences of nucleotide and amino acid of CTB are shown in Figure 2.5, and those of GFP-CTA<sub>2</sub> are shown in Figure 3.6.

#### DNA sequence of GFP-CTA<sub>2</sub>

ATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGT  
 AAACGGCCACAAGTTCAGCGTGTCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGA  
 AGTTCATCTGCACCACCGGCAAGCTGCCCCGTGCCCTGGCCCCACCCTCGTGACCACCCTGACCTACGGCG  
 TGCAGTGCTTCAGCCGCTACCCCGACCACATGAAGCAGCAGCACTTCTTCAAGTCCGCCATGCCCCGAAG  
 GCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGT  
 TCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATC  
 CTGGGGCACAAGCTGGAGTACAACCTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAAC  
 GGCATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGACGCTCGCCGACCACTAC  
 CAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCCGACAACCACTACCTGAGCACCCAGTCC  
 GCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGG  
 GATCACTCTCGGCATGGACGAGCTGTACAAG—*AGTAATACTTGCGATGAAAAACCCAAAGTCTAGGTGTAA*  
*AATTCTTGACGAATACCAATCTAAAGTAAAAGACAAATATTTTCAGGCTATCAATCTGATATTGATACATAATA*  
*GAATTAAGGATGAATTA*

#### Amino acid sequence of GFP-CTA<sub>2</sub>

MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTCLKFICTTGKLPVPWPTLVTTLTLYGVQCF  
 SRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEY  
 NYNSHNVYIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEK  
 RDHMLLEFVTAAGITLGMDELYK--*SNTCDEKQSLGVKFLDEYQSKVKRQIFSGYQSDIDTHNRIKDEL*

Figure 3.6 Sequences of nucleotide and amino acid of GFP-CTA<sub>2</sub>

(Italic parts are the sequences of CTA<sub>2</sub>)

### 3.3.2 Optimal expression time in the RTS cell-free system

Time-courses of CTB and GFP-CTA<sub>2</sub> expression in the RTS cell-free system are shown in Figure 3.7. Results showed that the expressed quantity of CTB or GFP-CTA<sub>2</sub> was increased with the increase in the expression time, and the expression maximized at the time of 24h. So the time of 24h was used as the optimal expression time hereafter.

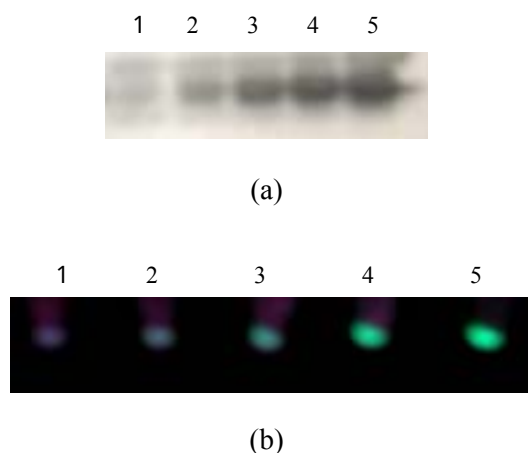


Figure 3.7 Time-course of protein expression in the RTS cell-free system

(a) Time-course of CTB expression (visualized by western blotting). Lane 1, CTB expressed for 4h; Lane 2, CTB expressed for 8h; Lane 3, CTB expressed for 12h; Lane 4, CTB expressed for 24h; Lane 5, CTB expressed for 36h. 5 $\mu$ l samples were applied. (b) Time-course of GFP-CTA<sub>2</sub> expression (visualized by fluorescence). Tube 1, GFP-CTA<sub>2</sub> expressed for 4h; Tube 2, GFP-CTA<sub>2</sub> expressed for 8h; Tube 3, GFP-CTA<sub>2</sub> expressed for 12h; Tube 4, GFP-CTA<sub>2</sub> expressed for 24h; Tube 5, GFP-CTA<sub>2</sub> expressed for 36h.

### 3.3.3 Expression of pentamer CTB

#### 3.3.3.1 Comparison of pentameric CTB expressed in *E. coli* and in the RTS cell-free system

It was found that the pentamer of commercial CTB was retained on SDS-PAGE after the treatment with SDS. Under the strict treatments with mercaptoethanol and heating at 90 °C for 5min, the pentamer of commercial CTB was separated into monomer.

Results in Figure 3.8 showed that nearly no pentameric CTB was visible by western blotting



when expressed in *E. coli*. Even in the case of expression of CTB possessing nearly the same structure with the natural one, the formation of pentamer was still difficult. This indicated that *E. coli* expression system should be, at least mainly, responsible for the poor formation of pentamer. Moreover, the dimer or trimer was very difficult to separate even with the treatments of mercaptoethanol and heating at 90 °C for 5min, which keeps unknown to our knowledge.

Figure 3.9 showed CTB expressed in the RTS cell-free system. In the expression of CTB and CTB-His×6, pentamers were formed steadily and completely. Even in the expression of His×6-22aa-CTB, at least half of the protein formed the pentamer.

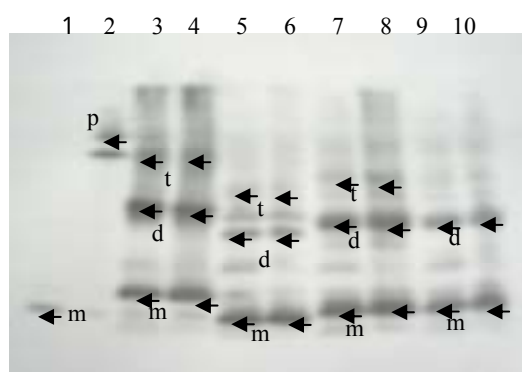


Figure 3.8 CTB expressed in *E. coli* (detected by western blotting)

Lane 1, Commercial CTB (+); Lane 2, Commercial CTB (-); Lane 3, His×6-22aa-CTB (+); Lane 4, His×6-22aa-CTB (-); Lane 5, CTB (+); Lane 6, CTB (-); Lane 7, His×6-CTB (+); Lane 8, His×6-CTB (-); Lane 9, CTB-His×6 (+); Lane 10, CTB-His×6 (-). Arrows indicated monomer or multimer of CTB. m, monomer; d, dimer; t, trimer; p, pentamer.

+, Samples were mixed in the sample buffer (two times concentrated) containing 12% β-mercaptoethanol as well as treated with heat at 90 °C for 5min. -, Samples were mixed in the 2×sample buffer without the addition of β-mercaptoethanol, and were not heated.

The formation of multimeric CTB expressed in *E. coli* and the RTS cell-free system is summarized in Table 3.6. It can be concluded that the RTS cell-free system expressed pentameric CTB successfully.

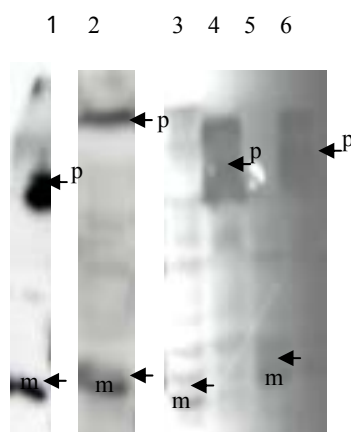


Figure 3.9 CTB expressed in the RTS cell-free system (detected by western blotting)

Lane 1, Commercial CTB (-); Lane 2, His×6-22aa-CTB (-); Lane 3, CTB (+); Lane 4, CTB (-); Lane 5, CTB-His×6 (+); Lane 6, CTB-His×6 (-). Arrows indicated monomer or multimer of CTB. m, monomer; p, pentamer. +, Samples were mixed in the sample buffer (two times concentrated) containing 12% β-mercaptoethanol as well as treated with heat at 90 °C for 5min. -, Samples were mixed in the 2×sample buffer without the addition of β-mercaptoethanol, and were not heated.

Table 3.6 Comparison of CTB expressed in *E. coli* and the RTS cell-free system

CTB structure (N→C)	Theoretical MW (kDa)	Sample treatment	Expressed CTB	
			<i>E. coli</i>	RTS cell-free system
His×6-22aa-CTB	15	+	monomer~trimer	not determined
		-	monomer~trimer	monomer and pentamer
CTB	11.6	+	monomer~trimer	monomer
		-	monomer~trimer	pentamer
His×6-CTB	12.5	+	monomer~trimer	not determined
		-	monomer~trimer	not determined
CTB-His×6	12.5	+	monomer~dimer	monomer
		-	monomer~dimer	pentamer

+, Samples were mixed in the 2×sample buffer containing 12% β-mercaptoethanol as well as treated with heat at 90 °C for 5min. -, Samples were mixed in the 2×sample buffer without the addition of β-mercaptoethanol, and were not heated.

3.3.3.2 Further verification of pentameric CTB expressed in the RTS cell-free system by GM<sub>1</sub>-ELISA

The linear relationship between the absorbance and the concentration of CTB is shown in Figure 3.10. The expression level of pentameric CTB in the RTS cell-free system is shown in Table 3.7.

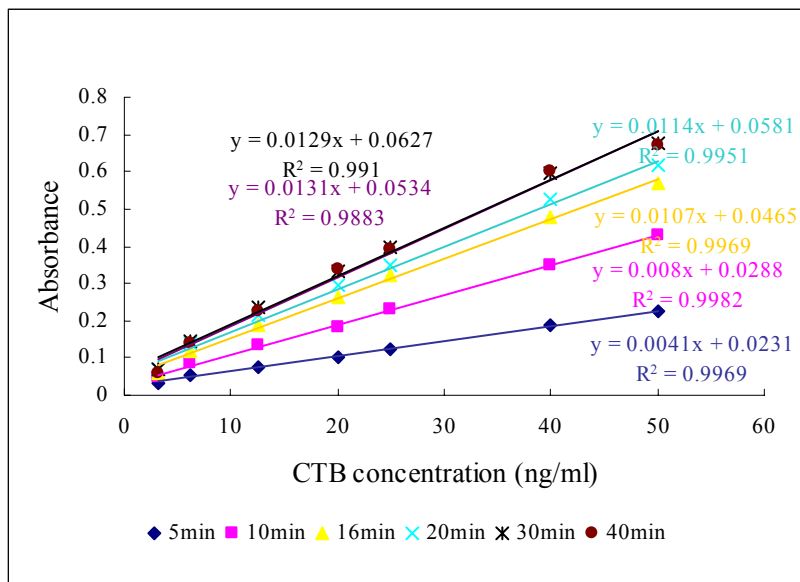


Figure 3.10 Linear relationship of absorbance versus concentration of CTB at the different color developing time

Table 3.7 Concentration measurement of pentameric CTB expressed in the RTS cell-free system

Sample	Dilution times	Concentration (ng/ml) determined at different color developing time						Mean concentration (μg/μl)
		5 min	10min	16min	20min	30min	40min	
CTB	2000×	49	38	39	40	39	37	0.128
	3000×	44	35	36	38	37	38	
	4000×	37	31	31	34	35	37	
	6000×	32	28	28	30	31	32	
CTB-His×6	2000×	33	29	30	32	32	33	0.109
	3000×	30	28	30	32	33	35	
	4000×	31	27	28	30	32	34	
	6000×	23	26	25	25	28	32	

Ratio of pentameric CTB or CTB-His×6 expressed in the RTS cell-free system was estimated by comparison with known concentrations of commercial CTB. The result of western blotting is shown in Figure 3.11. It was estimated that the bands of CTB and CTB-His×6 expressed in the RTS cell-free system were the same or larger than the band with 0.5μg of commercial CTB. Because 5μl of sample was applied, concentrations of these CTB are equivalent to or larger than 0.1μg/μl. Compared with the result from GM<sub>1</sub>-ELISA, the conclusion can be drawn that nearly 100% of CTB expressed in the RTS cell-free system formed pentamer.

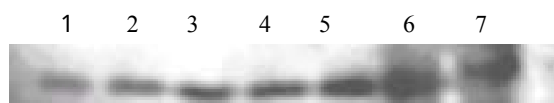


Figure 3.11 Ratio estimation of pentameric CTB expressed in the RTS cell-free system (detected by western blotting)

Lane 1, 0.1μg commercial CTB; Lane 2, 0.2μg commercial CTB; Lane 3, 0.3μg commercial CTB; Lane 4, 0.4μg commercial CTB; Lane 5, 0.5μg commercial CTB; Lane 6, CTB expressed in the RTS cell-free system (5μl); Lane 7, CTB-His×6 expressed in the RTS cell-free system (5μl). Samples were mixed in the 2×sample buffer containing 12% β-mercaptoethanol as well as treated with heat at 90 °C for 5min before loaded on SDS-PAGE.

According to the result obtained in our laboratory before [43], only 0.6% of His×6-22aa-CTB expressed in *E. coli* formed pentamer. But in the RTS cell-free system, CTB pentamer formed easily and completely. It indicates that the cell-free system has the superior ability in expressing properly folded proteins, suggesting its potential applications in characterization studies, structural and functional assays of proteins.

### 3.3.4 Investigation of the formation of holotoxin-like complex of GFP-CTA<sub>2</sub> and CTB

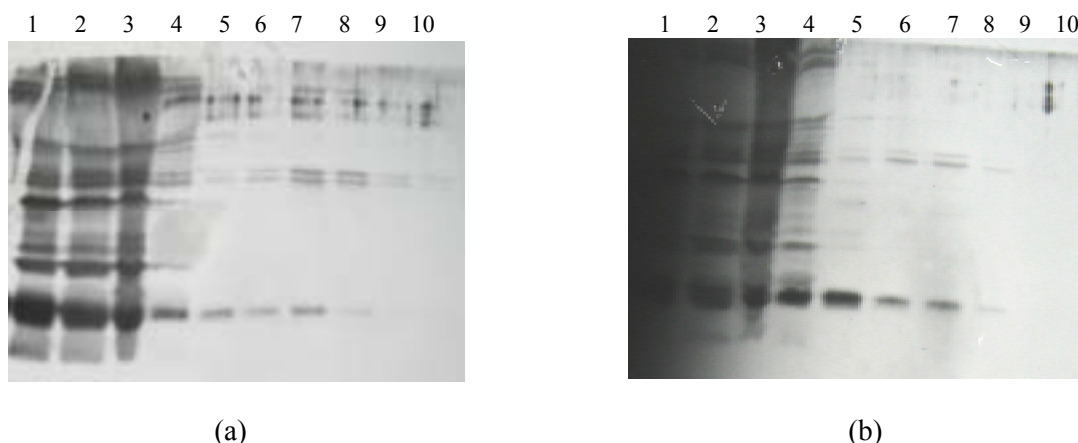


Figure 3.12 Purification of co-expressed and mixed GFP-CTA<sub>2</sub> and CTB

(a) Co-expressed GFP-CTA<sub>2</sub> and CTB (b) Mixed GFP-CTA<sub>2</sub> and CTB. Lane 1, CTB expressed in the cell-free system; Lane 2, Co-expressed or mixed GFP-CTA<sub>2</sub> and CTB; Lane 3, Flow-through (pooled, 20×concentrated); Lane 4, Washed fraction (pooled, 33×concentrated); Lane 5, Eluted fraction I (pooled, 8×concentrated); Lane 6, Eluted fraction II (the first eluted fraction, 2×concentrated); Lane 7, Eluted fraction II (the second eluted fraction, 2×concentrated); Lane 8, Eluted fraction II (the third eluted fraction, 2.5×concentrated); Lane 9, Eluted fraction II (the fourth eluted fraction, 2.5×concentrated); Lane 10, Eluted fraction III (pooled, 10×concentrated). Five µl protein samples were applied on SDS-PAGE.

It should be noticed that the mixture of GFP-CTA<sub>2</sub> and CTB cannot form the complex because CTA<sub>2</sub> only associates with the intermediate of CTB, but not the assembled CTB pentamer. Therefore, from the results shown in Figure 3.12, in spite of the detection of CTB in the purified fractions, nearly the same results were obtained from co-expression and mixture of GFP-CTA<sub>2</sub> and CTB. This indicated that the detected CTB was the nonspecifically absorbed one on the affinity column but not from the formed complex.

It was found that the fluorescence of GFP-CTA<sub>2</sub> was retained on SDS-PAGE after the treatment with SDS. When treated with mercaptoethanol and heating, the fluorescence of GFP-CTA<sub>2</sub> was lost.

GFP-CTA<sub>2</sub> visualized by fluorescence on SDS-PAGE and western blotting is shown in Figure 3.13. It was seen that GFP-CTA<sub>2</sub> was expressed as at least two products, which might be due to

the degradation or incomplete expression of protein. The expressed GFP-CTA<sub>2</sub> at different reaction time also had two bands on fluorescent SDS-PAGE (data not shown), which indicated the degradation or incomplete expression happened in the expression process, and was time-independent. The addition of protease inhibitor was not workable (data not shown).

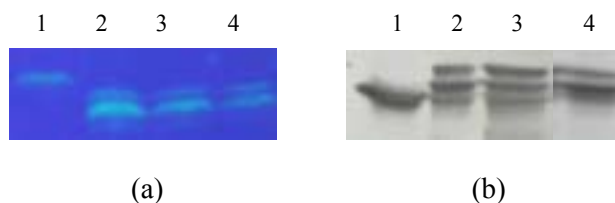


Figure 3.13 Visualization of GFP-CTA<sub>2</sub> expressed in the RTS cell-free system

(a) Visualized by fluorescence on SDS-PAGE (b) Visualized by western blotting. Lane 1, Positive control GFP (wild type); Lane 2, GFP-CTA<sub>2</sub>; Lane 3, Mixture of GFP-CTA<sub>2</sub> and CTB; Lane 4, Coexpression of GFP-CTA<sub>2</sub> and CTB.

In theory, GFP-CTA<sub>2</sub> (expressed from pIVEX2.4d, 35kDa) should have a higher molecular weight than that of positive control GFP (wild type, 28kDa). When they were treated with heat and mercaptoethanol, it was the case (Figure 3.13b). However, actually GFP-CTA<sub>2</sub> showed a lower apparent molecular weight than that of GFP on SDS-PAGE when both of them were not treated with mercaptoethanol and heating (Figure 3.13a). This indicated that the compact structure of GFP-CTA<sub>2</sub> might be due to the interaction between the segments of GFP and CTA<sub>2</sub>.

There are some assumptions about the failure in the formation of holotoxin-like complex between GFP-CTA<sub>2</sub> and CTB. These are: (1) Degradation or incomplete expression of GFP-CTA<sub>2</sub> made no complete CTA<sub>2</sub> available; (2) Hydrophobic region inside the cylindrical structure (shown in Figure 3.14) of GFP buried the hydrophobic CTA<sub>2</sub>; (3) Some chaperons function to facilitate and/or stabilize the formation of complex. To solve the problems, more stable and/or more hydrophilic model protein instead of GFP, or a flexible linker between GFP and CTA<sub>2</sub> is expected to be workable. Also, the functional chaperons have to be investigated.



Figure 3.14 Molecular structure of green fluorescence protein.

Eleven strands of  $\beta$ -sheet (green) form the walls of a cylinder, and short segments of  $\alpha$ -helices (blue) cap the top and bottom of the can and also provide a scaffold for the fluorophore which is near geometric center of the can. (from <http://www-bioc.rice.edu/Bioch/Phillips/Papers/gfpbio.html>)

### 3.4 Summary

Pentameric CTB was little expressed in *E. coli*, but expressed in the RTS cell-free system steadily and completely. This indicated that the cell-free system was a valuable tool and platform for the expression of structurally complex oligomeric proteins.

An attempt to assemble cholera toxin-like complex by coexpressing GFP-CTA<sub>2</sub> and CTB in the RTS cell-free system was done. Results revealed no formation of complex. More stable and/or more hydrophilic model protein instead of GFP, or a flexible linker between GFP and CTA<sub>2</sub> is expected to be workable for the formation of complex.

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## Acknowledgement

I wish to express my sincerest gratitude to my supervisor Prof. Keiichi Enomoto for his painstaking efforts and patience to direct me into the colorful world of biochemistry. Thanks him for not only providing me with well-equipped facilities and good quality reagents as well as continuous encouragement in my research, but also much help in my life. I am fortunate to have the chance to study in Japan, and most fortunately, to meet such a kind professor. I think the experience in the three years is unforgettable in my life, and is the invaluable wealth for my future.

Appreciation is given to Prof. Yasuo Mukohata, Prof. Nobuya Matsumoto, Associate Prof. Osamu Ariga, Prof. Takeshi Ohama and Associate Prof. Sakae Horisawa for their constructive comment and suggestions in my research. Grateful acknowledgement is given to Prof. Keiko Udaka of Kochi University for her valuable suggestions in the future experiment of T-cell proliferation of fusion proteins. I also want to express my sincere gratitude to Dr. Mitsuhiro Itaya of Mitsubishi Kagaku Institute of Life Sciences for his patient instruction and kindly providing of vectors and *Bacillus subtilis* strains in the *B. subtilis* experiment. I'm also grateful to Prof. Takeshi Honda of Osaka University for his kindly providing of cholera toxin gene of *Vibrio cholerae*.

I thank Mr Shuichi Yada, Mr Takenari Aharen, Mr Takahiro Kobata, Ms Meimei Han, Mr Yujiro Beppu, Ms Yuko Okuda, Ms Minako Eguchi and Mr Kentaro Ito for their valuable suggestions and pleasant technical collaboration during the different phase in my research. Especial appreciation should be given to Mr Kakushi Hosokawa for his kind help in the printing of conference materials, which was troublesome and time-consuming. I would also like to express my appreciation to other members in my research laboratory, including Ms Yi Wang and Ms Satoka Okaue.

I wish to thank Prof. Masaoki Furue and Prof. Kazuya Kobiro for their kind invitation to have the enjoyable time with their family. I also want to express my appreciation to Prof. Hiroshi Tamaki and Prof. Kiichiro Nishihara for their warm-hearted help. I would like to thank the personnel in IRC for their kindness to the international students. I also want to express my

thanks to Japanese and Chinese friends for their direct and indirect help during my study in Japan.

I would like to give the appreciation to my friends, Ms Danhua Liu, Ms Xiuli Zhao and Ms Li Zhang for their continuous support and encouragement. I really feel lucky to share joys and sorrows in my life with them. I also want to express my deepest gratitude to my parents for understanding and supporting me in my different life phases.

# Appendix

## I Abbreviations

CT	cholera toxin
CTB	cholera toxin B subunit
CTA	cholera toxin A subunit
Cry j	Allergen protein from the pollen of <i>Cryptomeria japonica</i>
GFP	green fluorescent protein
BSA	bovine serum albumin
IgE	immunoglobulin E
MHC	major histocompatibility complex
HLA	human leukocyte antigens
DNA	deoxyribonucleic acid
PCR	polymerase chain reaction
FW	forward
RV	reverse
aa	amino acid
<i>E. coli</i>	<i>Escherichia coli</i>
<i>B. subtilis</i>	<i>Bacillus subtilis</i>
RBS	ribosome-binding site
ORF	open reading frame
T-vector	T-extended vector
LB	Luria-Bertani
IPTG	isopropyl- $\beta$ -D-thiogalactopyranoside
X-gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside
Amp	ampicillin
Cm	chloramphenicol

Kana	kanamycin
Tc	tetracycline
Bs	blasticidin S
Tris	tris (hydroxymethyl) aminomethane
PBS	phosphate buffered saline
His×6	hexahistidine
TCA	trichloroacetic acid
Ni-NTA	nickel-nitrilotriacetic acid
SDS	sodium dodecyl sulfate
APS	ammonium persulfate
TEMED	N’N’-bis-methylene-acrylamide
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
PVDF	polyvinylidene difluoride
ELISA	enzyme-linked immunosorbent assay
kDa	kilodalton
RTS	rapid translation system
CECF	continuous exchange cell-free



## II Reagents preparation

### 1. DNA operation

#### a. 10×TAE Buffer

Tris	48.4g
Acetic acid	11ml
EDTA solution (0.25M)	40ml
Distilled water	to 1000ml (adjusted to pH 7.8)

#### b. 5×TBE Buffer

Tris	54g
Boric acid	27.5g
EDTA • 2Na	3.7g
Distilled water	to 1000ml

#### c. TE Buffer

Tris	10mM
EDTA	1mM (pH 8.0)

### 2. Bacterial cultivation and protein expression

#### a. LB medium

Tryptone	10g
Yeast Extract	5g
NaCl	10g
Agar	15g (for liquid medium, agar is not needed)
Distilled water	to 1000ml (adjusted to pH 7.2 and autoclaved)

b. 2M Mg solution (1M MgCl<sub>2</sub>/1M MgSO<sub>4</sub>, filter-sterilized)

MgCl <sub>2</sub> • 6H <sub>2</sub> O	2.033g
MgSO <sub>4</sub> • 7H <sub>2</sub> O	2.4648g
Distilled water	to 10ml

c. SOB medium

Tryptone	20g	} (autoclaved and cooled to the room temperature before the addition of 2M Mg solution)
Yeast extract	5g	
NaCl	0.585g	
KCl	0.186g	
Distilled water	to 1000ml	
2M Mg solution	10ml	

d. SOC medium

SOB medium	1000ml
glucose solution (2M)	10ml (filter-sterilized and stored at 4 °C)

e. Ampicillin stock solution (100mg/ml, stored at -20 °C)

Ampicillin sodium	5g
Distilled water	to 50ml

f. Kanamycin stock solution (25mg/ml, stored at -20 °C)

Kanamycin sulfate	1.25g
Distilled water	to 50ml

g. Tetracycline stock solution (5mg/ml, stored at -20 °C and protected from light)

Tetracycline hydrochloride	0.25g
Ethanol	to 50ml

h. Chloramphenicol stock solution (5mg/ml, stored at -20 °C and protected from light)

Chloramphenicol	0.25g
Ethanol	to 50ml

i. Blasticidin S stock solution (250mg/ml, stored at -20 °C)

Blasticidin S hydrochloride	25mg
Distilled water	to 100µl

j. IPTG stock solution (100mM, stored at -20 °C)

IPTG	23.8mg
Distilled water	to 1ml

k. X-gal stock solution (40mg/ml, stored at -20 °C and protected from light)

X-gal	400mg
Dimethylformamide	to 10ml

### 3. Protein separation (SDS-PAGE)

a. Acrylamide/bis solution (30%, stored at 4 °C and protected from light)

Acrylamide	29.2g
TEMED	0.8g
Distilled water	to 100ml

b. 1.5M Tris-HCl buffer (pH 8.8, stored at 4 °C)

Tris	18.15g
Distilled water	to 100ml

c. 0.5M Tris-HCl buffer (pH 6.8, stored at 4 °C)

Tris	6g
Distilled water	to 100ml

d. SDS solution (10%, stored at room temperature)

SDS (sodium dodecyl sulfate)	10g
Distilled water	to 100ml

e. APS solution (10%, freshly prepared)

APS	0.1g
Distilled water	1ml

f. 2×sample buffer

0.5M Tris-HCl buffer (pH 6.8)	2ml
SDS solution (10%)	4ml
β-mercaptoethanol	1.2ml
Glycerol	2ml
Distilled water	0.8ml
Bromophenol blue solution (1%)	some drops

g. 5×electrode buffer (pH 8.3)

Tris	15g
Glycine	72g
SDS	5g
Distilled water	to 1000ml

h. Destaining solution

Methanol	1200ml
Acetic acid	300ml
Distilled water	1500ml

i. Staining solution (0.1%)

Coomassie Blue R-250	0.3g
Destaining solution	to 300ml

4. Immunodetection of protein (western blotting)

a. Blotting buffer

Tris	12g
Glycine	14.4g
Distilled water	800ml
Methanol	200ml

b. 10×PBS buffer (stored at 4 °C)

NaCl	80g
Na <sub>2</sub> HPO <sub>4</sub> • 12H <sub>2</sub> O	29g
(or Na <sub>2</sub> HPO <sub>4</sub> • 7H <sub>2</sub> O	(or 22g or 11g)
or anhydrous Na <sub>2</sub> HPO <sub>4</sub> )	
KCl	2g
KH <sub>2</sub> PO <sub>4</sub>	2g
Distilled water	to 1000ml

c. Blocking buffer (1%)

Milk powder	0.5g
1×PBS buffer	to 50ml

d. PBS-T buffer (0.5%)

Tween 20	5g
1×PBS buffer	to 1000ml

5. Immunodetection of protein (ELISA)

a. GM<sub>1</sub>-ganglioside stock solution (20μg/ml, stored at -20 °C)

GM <sub>1</sub> -ganglioside	1mg
Methanol	to 50ml

b. BSA/PBS stock solution (10%, stored at 4 °C)

BSA	5g	} (dissolved at 50 °C, 0.22μm-filtrated)
Distilled water	45ml	
10×PBS buffer	5ml	

c. Blocking buffer (1%)

BSA/PBS stock solution (10%)	1ml
1×PBS buffer	9ml

d. Washing buffer

Tween 20	0.25g	} : =99:1
10×PBS buffer	49.5ml	
Distilled water	to 495ml	
BSA/PBS stock solution (10%)	5ml	